(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 June 2008 (12.06.2008)

PCT

(10) International Publication Number
WO 2008/070152 A2

(51) International Patent Classification:
C12Q 1/48 (2006.01)

(21) International Application Number:
PCT/US2007/024992

(22) International Filing Date:
6 December 2007 (06.12.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/873,753 6 December 2006 (06.12.2006) US

(71) Applicant *(for all designated States except US)*: **ALBERT EINSTEIN COLLEGE OF MEDICINE OF YESHIVA UNIVERSITY** [US/US]; 1300 Morris Park Avenue, Bronx, NY 10461 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **LAWRENCE, David, S.** [US/US]; 102 Huntington Drive, Chapel Hill, NC 27514 (US). **SHARMA, Vyas** [IN/US]; 1100 West NC-54 Hwy BYP, Apt 25F, Chapel Hill, NC 27516 (US). **AGNES, Richard, S.** [US/US]; 2400 Kenilworth Road, Cleveland Heights, OH 44106 (US).

(74) Agents: **MILLER, Alan, D.** et al.; Amster, Rothstein & Ebenstein LLP, 90 Park Avenue, New York, NY 10016 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

WO 2008/070152 A2

(54) Title: DEEPLY QUENCHED ENZYME SENSORS AND BINDING SENSORS

(57) Abstract: Sensors for detecting enzyme activity are provided that include a substrate module comprising a substrate for the enzyme of interest and a fluorescent label, a quencher, and a detection module. The detection module binds to the substrate module either before or after the enzyme acts on the substrate and sequesters the label from the quencher, resulting in an increased signal from the label. Sensors for detecting protein-protein interactions are also provided that include a quencher and a labeled first polypeptide. Binding of the first polypeptide to a second polypeptide sequesters the label from the quencher, resulting in an increased signal from the label. Methods using the sensors to detect enzyme activity and to screen for compounds affecting enzyme activity or to detect protein-protein interactions and to screen for compounds affecting protein-protein interactions, respectively, are also described.

DEEPLY QUENCHED ENZYME SENSORS AND BINDING SENSORS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 60/873,753, filed on December 6, 2006, the content of which is hereby incorporated by reference.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with United States government support under Grant Nos. GM067198 and NS048406 from the National Institutes of Health. Accordingly, the United States government has certain rights in this invention.

FIELD OF THE INVENTION

[0003] The invention relates to sensors for detecting enzyme activity and uses thereof. The enzyme sensors include a substrate module comprising a substrate for the enzyme and a label, a detection module, and a quencher. Binding of the substrate module to the detection module sequesters the label from the quencher, resulting in an increase in signal from the label. The invention also relates to sensors for detecting protein-protein interactions and uses thereof.

BACKGROUND OF THE INVENTION

[0004] Detection of enzyme activity is a necessary step in a wide variety of clinical and basic research applications. For example, in one approach to identifying lead compounds in drug discovery programs, a large number of compounds are screened for activity as inhibitors or activators of a particular enzyme's activity. As just one example, since abnormal protein phosphorylation has been implicated in a number of diseases and pathological conditions including arthritis, cancer, diabetes, and heart disease, screening for compounds capable of modulating the activity of various protein kinases or protein phosphatases can produce lead compounds for evaluation in treatment of these conditions (see, e.g., Ross et al. (2002) "A non-radioactive method for the assay of many serine/threonine-specific protein kinases" Biochem. J. 366:977-998 and references therein).

[0005] Simple and reproducible methods for qualitative and/or quantitative detection of enzyme activity are thus desirable, for drug discovery and a wide variety of other applications. Among other benefits, the present invention provides sensors for detecting enzyme activity, as well as related methods for detection of enzyme activity and for screening for compounds affecting enzyme activity.

DEFINITIONS

[0006] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. The following definitions supplement those in the art and are directed to the current application and are not to be imputed to any related or unrelated case, e.g., to any commonly owned patent or application. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. Accordingly, the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0007] As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a molecule" includes a plurality of molecules, and the like.

[0008] The term "about" as used herein indicates the value of a given quantity varies by +/-10% of the value, or optionally +/- 5% of the value, or in some embodiments, by +/- 1% of the value so described.

[0009] An "acetyltransferase" is an enzyme that catalyzes the transfer of an acetyl group from one molecule to another. A "lysine acetyltransferase" transfers an acetyl group, typically from acetyl coenzyme A, to the ε-amino group of a lysine residue in a protein. A "histone acetyltransferase" transfers an acetyl group to a histone, e.g., to the ε-amino group of a lysine residue in the histone.

[0010] An "amino acid sequence" is a polymer of amino acid residues (a protein, polypeptide, etc) or a character string representing an amino acid polymer, depending on context.

[0011] As used herein, an "antibody" is a protein comprising one or more polypeptides substantially or partially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable

region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes IgG, IgM, IgA, IgD and IgE, respectively. A typical immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these light and heavy chains respectively. Antibodies exist as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'2, a dimer of Fab which itself is a light chain joined to VH-CH1 by a disulfide bond. The F(ab)'2 may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the (Fab')2 dimer into a Fab' monomer. The Fab' monomer is essentially a Fab with part of the hinge region (*see* "Fundamental Immunology," WE Paul, ed, Raven Press, NY (1999), for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, includes antibodies or fragments either produced by the modification of whole antibodies or synthesized *de novo* using recombinant DNA methodologies. Antibodies include multiple or single chain antibodies, including single chain Fv (sFv or scFv) antibodies in which a variable heavy and a variable light chain are joined together (directly or through a peptide linker) to form a continuous polypeptide.

[0012] An "aptamer" is a nucleic acid capable of interacting with a ligand. An aptamer can be, e.g., a DNA or RNA, and can be eg a chemically synthesized oligonucleotide. The ligand can be any natural or synthetic molecule, including, e.g., the first or second state of a substrate.

[0013] A "caging group" is a moiety that can be employed to reversibly block, inhibit, or interfere with the activity (e.g., the biological activity) of a molecule (e.g., a polypeptide, a nucleic acid, a small molecule, a drug, etc.). The caging groups can, e.g., physically trap an active molecule inside a framework formed by the caging groups. Typically, however, one or more caging groups are associated (covalently or noncovalently) with the molecule but do not necessarily surround the molecule in a physical cage. For example, a single caging group

covalently attached to an amino acid side chain required for the catalytic activity of an enzyme can block the activity of the enzyme. The enzyme would thus be caged even though not physically surrounded by the caging group. As another example, covalent attachment of a single caging group to an amino acid side chain that is phosphorylated by a kinase in a kinase substrate can block phosphorylation of that substrate by the kinase. Caging groups can be, e.g., relatively small moieties such as carboxyl nitrobenzyl, 2-nitrobenzyl, nitroindoline, hydroxyphenacyl, DMNPE, or the like, or they can be, e.g., large bulky moieties such as a protein or a bead. Caging groups can be removed from a molecule, or their interference with the molecule's activity can be otherwise reversed or reduced, by exposure to an appropriate type of uncaging energy and/or exposure to an uncaging chemical, enzyme, or the like.

[0014] A "photoactivatable" or "photoactivated" caging group is a caging group whose blockage of, inhibition of, or interference with the activity of a molecule with which the photoactivatable caging group is associated can be reversed or reduced by exposure to light of an appropriate wavelength. For example, exposure to light can disrupt a network of caging groups physically surrounding the molecule, reverse a noncovalent association with the molecule, trigger a conformational change that renders the molecule active even though still associated with the caging group, or cleave a photolabile covalent attachment to the molecule, etc.

[0015] A "photolabile" caging group is one whose covalent attachment to a molecule is reversed (cleaved) by exposure to light of an appropriate wavelength. The photolabile caging group can be, e.g., a relatively small moiety such as carboxyl nitrobenzyl, 2-nitrobenzyl, nitroindoline, hydroxyphenacyl, DMNPE, or the like, or it can be, e.g., a relatively bulky group (eg a macromolecule, a protein) covalently attached to the molecule by a photolabile linker (e.g., a polypeptide linker comprising a 2-nitrophenyl glycine residue).

[0016] A "Dab residue" is an (L)-2,4-diaminobutyric acid residue.

[0017] A "Dap residue" is an (L)-2,3-diaminopropionic acid residue.

[0018] An "enzyme" is a biological macromolecule that has at least one catalytic activity (ie, that catalyzes at least one chemical reaction). An enzyme is typically a protein, but can be, e.g., RNA. Known protein enzymes have been grouped into six classes (and a number of subclasses and sub-subclasses) under the Enzyme Commission classification scheme (*see*, e.g. the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology enzyme nomenclature pages, on the world wide web at www (dot) chem (dot) qmul (dot) ac (dot) uk/iubmb/enzyme), namely, oxidoreductase, transferase, hydrolase, lyase, ligase, or isomerase. The activity of an enzyme can be "assayed," either qualitatively (e.g., to

determine if the activity is present) or quantitatively (e.g., to determine how much activity is present or kinetic and/or thermodynamic constants of the reaction).

[0019] A "kinase" is an enzyme that catalyzes the transfer of a phosphate group from one molecule to another. A "protein kinase" is a kinase that transfers a phosphate group to a protein, typically from a nucleotide such as ATP. A "tyrosine protein kinase" (or "tyrosine kinase") transfers the phosphate to a tyrosine side chain (e.g., a particular tyrosine), while a "serine/threonine protein kinase" ("serine/threonine kinase") transfers the phosphate to a serine or threonine side chain (e.g., a particular serine or threonine).

[0020] A "label" is a moiety that facilitates detection of a molecule. Fluorescent labels are preferred labels in the context of the invention. Many labels are known in the art and commercially available and can be used in the context of the invention.

[0021] An "environmentally sensitive label" is a label whose signal changes when the environment of the label changes. For example, the fluorescence of an environmentally sensitive fluorescent label changes when the hydrophobicity, pH, and/or the like of the label's environment changes (e.g., upon binding of the molecule with which the label is associated to another molecule such that the label is transferred from an aqueous environment to a more hydrophobic environment at the molecular interface).

[0022] A "methyltransferase" is an enzyme that catalyzes the transfer of a methyl group from one molecule to another. A "protein lysine methyltransferase" transfers a methyl group to the ε-amino group of a lysine residue in a protein. A "histone methyltransferase" transfers a methyl group, e.g., from S-adenosyl methionine, to a histone; a "histone lysine methyltransferase" transfers a methyl group to a lysine residue in a histone, while a "histone arginine methyltransferase" transfers a methyl group to an arginine residue in a histone.

[0023] A "modulator" enhances or inhibits an activity of an enzyme or protein (e.g., a catalytic activity of an enzyme), either partially or completely. An "activator" enhances the activity (whether moderately or strongly). An "inhibitor" inhibits the activity (e.g., an inhibitor of an enzyme attenuates the rate and/or efficiency of catalysis), whether moderately or strongly. A modulator can be, e.g., a small molecule, a polypeptide, a nucleic acid, etc.

[0024] The term "nucleic acid" encompasses any physical string of monomer units that can be corresponded to a string of nucleotides, including a polymer of nucleotides (e.g., a typical DNA or RNA polymer), peptide nucleic acids (PNAs), modified oligonucleotides (e.g., oligonucleotides comprising nucleotides that are not typical to biological RNA or DNA in solution, such as 2'-O-methylated oligonucleotides), and the like. The nucleotides of the nucleic acid can be deoxyribonucleotides, ribonucleotides or nucleotide analogs, can be

natural or non-natural, and can be unsubstituted, unmodified, substituted or modified. The nucleotides can be linked by phosphodiester bonds, or by phosphorothioate linkages, methylphosphonate linkages, boranophosphate linkages, or the like. The nucleic acid can additionally comprise non-nucleotide elements such as labels, quenchers, blocking groups, or the like. A nucleic acid can be e.g., single-stranded or double-stranded. Unless otherwise indicated, a particular nucleic acid sequence of this invention encompasses complementary sequences, in addition to the sequence explicitly indicated.

[0025] A "phosphatase" is an enzyme that removes a phosphate group from a molecule. A "protein phosphatase" removes the phosphate group from an amino acid side chain in a protein. A "serine/threonine-specific protein phosphatase" removes the phosphate from a serine or threonine side chain (e.g., a particular serine or threonine), while a "tyrosine-specific protein phosphatase" removes the phosphate from a tyrosine side chain (e.g., a particular tyrosine).

[0026] A "polypeptide" is a polymer comprising two or more amino acid residues (e.g., a peptide or a protein). The polymer can additionally comprise non-amino acid elements such as labels, blocking groups, or the like and can optionally comprise modifications such as glycosylation or the like. The amino acid residues of the polypeptide can be natural or non-natural and can be unsubstituted, unmodified, substituted or modified.

[0027] A "quencher" is a moiety that alters a property of a label (typically, a fluorescent label) when it is in proximity to the label. For example, the quencher can quench (reduce the intensity of) a fluorescent emission from a fluorescent label when it is proximal to the label as compared to when not proximal to the label. A quencher can be, e.g., an acceptor fluorophore that operates via energy transfer and re-emits the transferred energy as light. Other similar quenchers, called "dark quenchers," do not re-emit transferred energy via fluorescence.

[0028] A "substrate" is a molecule on which an enzyme acts. The substrate is typically supplied in a first state on which the enzyme acts, converting it to a second state. The second state of the substrate is then typically released from the enzyme.

[0029] "Uncaging energy" is energy that removes one or more caging groups from a caged molecule (or otherwise reverses the caging groups' blockage of the molecule's activity). As appropriate for the particular caging group(s), uncaging energy can be supplied, e.g., by light, sonication, a heat source, a magnetic field, or the like.

[0030] A variety of additional terms are defined or otherwise characterized herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] Figure 1. Fluorescence fold-change as a function of time in the presence of Rose Bengal/**P5** (upper trace), Aniline Blue WS/**P9** (middle trace), or Ponceau S/**P2** (lower trace) pairs [PKA] were chosen for each pair so that the reaction would be completed within 3 minutes [Rose Bengal/**P5** (25 μM); Aniline Blue WS/**P9** (0.7 μM); Ponceau S/**P2** (10 nM)].

[0032] Figure 2. Percent fluorescent quenching of peptide **P2** as a function of the concentration of Dyes **1** – **10**.

[0033] Figure 3A-3B. Percent fluorescent quenching of pyrene fluorescence in (A) peptide **P5** with Rose Bengal dye (upper trace) and peptide **P9** with Aniline Blue WS dye (lower trace), and (B) phosphorylated peptide **P5** and Rose Bengal.

[0034] Figure 4. Fluorescence as a function of [peptide **P5**] before (solid, upper trace) and after (dotted, lower trace) background correction.

[0035] Figure 5. PKA-induced fluorescence change of the Rose Bengal/peptide **P5** pair in the presence and absence (lower trace) of 14-3-3τ.

[0036] Figure 6A-6D. Fractional PKA activity versus log [inhibitor] for (A) H9•HCl at 10 μM ATP, (B) H9•HCl at 1 mM ATP, (C) PKI (14-22) using Deep Quench method, (D) PKI (14-22) using the standard radioactive ATP method.

[0037] Figure 7. PKA-induced fluorescence change of peptide **P1** – **P11** in the presence of the ten lead quencher dyes. Peptide concentration was fixed at 5 μM and a 5-, 10-, 25-, and 50-fold excess of quencher was employed. Darkest shading = 8-fold change and above.

[0038] Figure 8. Enhancement in fluorescence using substrate **P12.** Using **P12** as the substrate module with an Acid Green 27 quencher and a 14-3-3 detection module, a 225-fold enhancement in fluorescence was observed.

DETAILED DESCRIPTION

[0039] In one aspect, the invention provides a variety of sensors for detecting enzyme activity. In one class of embodiments, the sensor includes a substrate module, a quencher, and a detection module. The substrate module includes a substrate for the enzyme of interest and a fluorescent label. The detection module binds to the substrate module either before or after the enzyme acts on the substrate and sequesters the label from the quencher, resulting in an increased signal from the label. Compositions, kits, and systems including the sensors or components thereof and methods for using the sensors to detect enzyme activity and to screen for compounds affecting enzyme activity are described.

[0040] In another aspect, the invention provides a variety of sensors for detecting protein-protein interactions. In one class of embodiments, the binding sensor includes a quencher and a labeled polypeptide that comprises a first polypeptide and a label. Binding of the first polypeptide to a second polypeptide sequesters the label from the quencher, resulting in an increased signal from the label. Compositions, kits, and systems including the binding sensors or components thereof and methods for using the sensors to detect protein-protein interactions and to screen for compounds affecting protein-protein interactions are described.

ENZYME SENSORS

[0041] A first general class of embodiments provides a composition including a sensor for detecting an activity of an enzyme. The sensor comprises a substrate module, a detection module, and a quencher. The substrate module includes a substrate for the enzyme, wherein the substrate is in a first state on which the enzyme can act, thereby converting the substrate to a second state, and a fluorescent label. The detection module binds to the substrate module when the substrate is in the first state or when the substrate is in the second state. Binding of the detection module to the substrate module results in an increased intensity of fluorescent emission from the label, since the label is at least partially sequestered from the quencher. In one aspect, the quencher is not covalently bound to the substrate module or to the detection module. The composition optionally includes the enzyme.

[0042] The substrate and detection modules can be part of a single molecule. More typically, however, the substrate module comprises a first molecule and the detection module comprises a second molecule. For example, the substrate module can comprise a first polypeptide and the detection module a second polypeptide. It is worth noting that the substrate module can comprise essentially any suitable substrate, for example, one or more of an amino acid, a polypeptide, a nitrogenous base, a nucleoside, a nucleotide, a nucleic acid, a carbohydrate, a lipid, or the like. The substrate is optionally a specific substrate (acted on only by a single type of catalytic molecule, e.g., under a defined set of reaction conditions), or a generic substrate (acted on by more than one member of a class of catalytic molecules). Similarly, the detection module can comprise essentially any molecule that can bind the first or second state of the substrate, for example, a polypeptide, an aptamer, or the like.

[0043] The enzyme whose activity is to be detected can be essentially any enzyme. For example, the enzyme can be a transferase, or it can be an oxidoreductase, hydrolase, lyase, ligase, or isomerase. In one embodiment, the enzyme catalyzes a posttranslational modification of a polypeptide, for example, phosphorylation, acetylation, methylation, ubiquitination, sumoylation, glycosylation, prenylation, myristoylation, farnesylation,

attachment of a fatty acid, attachment of a GPI anchor, nucleotidylation (e.g., ADP-ribosylation), or the like. For example, the enzyme can be a transferase from any one of EC subclasses 2.1-2.9 (e.g., a glycosyltransferase, protein farnesyltransferase, or protein geranylgeranyltransferase), a ligase from any one of EC subclasses 6.1-6.6 (e.g., a ubiquitin transferase or ubiquitin-conjugating enzyme), or a hydrolase from any one of EC subclasses 3.1-3.13 (e.g., a phosphatase or glycosylase). The enzyme is optionally an enzyme that does not cleave its substrate (that is, optionally conversion of the substrate from the first state to the second state does not involve cleavage of the substrate by the enzyme).

[0044] In one preferred class of embodiments, the enzyme is a protein kinase. The substrate is therefore a substrate for a protein kinase, e.g., a polypeptide substrate for the kinase. In this class of embodiments, the substrate in the first state is unphosphorylated (not phosphorylated), and the substrate in the second state is phosphorylated. In some embodiments, the detection module binds to the substrate module when the substrate is in the first state; in other embodiments, the detection module binds to the substrate module when the substrate is in the second state (ie, the detection module binds to the phosphorylated substrate). It is worth noting that, in this class of embodiments as well as other embodiments herein, while the detection module can bind to the substrate in either the first state or the second state, embodiments in which the detection module binds to the substrate in the second state are generally preferable since in these embodiments the detection module is not competing with the enzyme for the substrate.

[0045] In one class of embodiments, the protein kinase is a serine/threonine protein kinase. The detection module is optionally, e.g., a polypeptide, an aptamer, or the like that recognizes the phosphorylated serine and/or threonine substrate. For example, the detection module can include a 14-3-3, FHA, WD40, WW, Vhs, HprK, DSP, KIX, MH2, PKI, API3, ARM, cyclin, CDI, or GlgA domain, or an antibody. The substrate and detection modules optionally comprise distinct polypeptides.

[0046] In one exemplary class of embodiments, the substrate module comprises a polypeptide substrate comprising amino acid sequence $X^{-4}\,R^{-3}\,R^{-2}\,X^{-1}\,S^{0}\,X^{+1}\,X^{+2}$ (SEQ ID NO:13); where X^{-4} and X^{+2} are independently selected from the group consisting of an amino acid residue and an amino acid residue comprising the fluorescent label; and where X^{-1} and X^{+1} are independently selected from the group consisting of a hydrophobic amino acid residue (e.g., Phe, Leu, Ile, etc) and an amino acid residue comprising the fluorescent label. The label is optionally attached to one of X^{-4}, X^{-1}, X^{+1} and X^{+2}, or to a residue or other moiety

N-terminal of X^{-4} or C-terminal of X^{+2}. The composition optionally includes a cAMP-dependent protein kinase (PKA) that can phosphorylate S^0.

[0047] For example, the substrate module can be any one of **P1-P12** (which are described in the Examples sections herein below), or it can comprise the amino acid sequence of any one of **P1-P12** (SEQ ID NOs: 1-12) and have a label (e.g., pyrene or a coumarin derivative) attached to the corresponding residue. The termini of the polypeptide are optionally free or modified; for example, the N-terminus can be free or acetylated and/or the C-terminus can be a free carboxyl or a C-terminal amide. In a few specific examples, the detection module is a 14-3-3 domain, and the substrate module is **P5** and the quencher is Rose Bengal, the substrate module is **P9** and the quencher is Aniline Blue WS, the substrate module is **P2** and the quencher is Ponceau S, or the substrate module is **P12** and the quencher is Acid Green 27. A number of additional exemplary sensors are described in the Examples section below.

[0048] In another class of embodiments, the protein kinase is a tyrosine protein kinase. The detection module is optionally, e.g., a polypeptide, an aptamer, or the like that recognizes the phosphorylated tyrosine substrate. For example, the detection module can include an SH2 domain, an FHA domain, a PTB (phosphotyrosine binding) domain, or an antibody. The substrate and detection modules optionally comprise distinct polypeptides.

[0049] Substrate and/or detection modules for use in the tyrosine protein kinase sensors are optionally adapted from those described in U.S. patent application 11/366,221 filed March 1, 2006 entitled "Enzyme sensors including environmentally sensitive or fluorescent labels and uses thereof" by David S Lawrence et al. Thus, in one exemplary class of embodiments, the fluorescent label is an environmentally sensitive fluorescent label; the substrate module includes a polypeptide comprising amino acid sequence $X^{-4}X^{-3}X^{-2}X^{-1}Y^{0}X^{+1}X^{+2}X^{+3}X^{+4}X^{+5}$ (SEQ ID NO:14); where X^{-4}, X^{-3}, and X^{-2} are independently selected from the group consisting of D, E, and an amino acid residue comprising the environmentally sensitive label; X^{-1} and X^{+3} are independently selected from the group consisting of: A, V, I, L, M, F, Y, W, and an amino acid residue comprising the environmentally sensitive label; X^{+1}, X^{+2}, X^{+4}, and X^{+5} are independently selected from the group consisting of: an amino acid residue (e.g., a naturally occurring amino acid residue) and an amino acid residue comprising the environmentally sensitive label; and at least one of X^{-4}, X^{-3}, X^{-2}, X^{-1}, X^{+1}, X^{+2}, X^{+3}, X^{+4}, and X^{+5} is an amino acid residue comprising the environmentally sensitive label; and the detection module optionally comprises an SH2 domain. In other embodiments, the protein kinase can be, e.g., a histidine kinase, an asp/glu kinase, or an arginine kinase.

[0050] The phosphopeptide binding domains noted above, as well as other phosphopeptide binding domains, have been well described in the literature. For example, the specificity of various SH2 domains for sequences surrounding the phosphorylated tyrosine residue has been determined. See, e.g., a list of phosphopeptide binding domains at folding (dot) cchmc (dot) org/online/SEPdomaindatabase (dot) htm; a list of protein interaction domains at www (dot) mshri (dot) on (dot) ca/pawson/domains (dot) html; a list of protein domains at www (dot) cellsignal (dot) com/reference/domain/index (dot) asp, which includes consensus binding sites, exemplary peptide ligands, and exemplary binding partners, e.g., for SH-2, 14-3-3, PTB, and WW domains; Kuriyan and Cowburn (1997) "Modular peptide recognition domains in eukaryotic signaling" Annu. Rev. Biophys. Biomol. Struct. 26:259-288; Sharma et al. (2002) "Protein-protein interactions: Lessons learned" Curr. Med. Chem. - Anti-Cancer Agents 2:311-330; Pawson et al. (2001) "SH2 domains, interaction modules and cellular wiring" Trends Cell. Biol. 11:504-11; Forman-Kay and Pawson (1999) "Diversity in protein recognition by PTB domains" Curr. Opin. Struct. Biol. 9:690-5; and Fu et al. (2000) "14-3-3 Proteins: Structure, Function, and Regulation" Annual Review of Pharmacology and Toxicology 40:617-647. A large number of such domains from a variety of different proteins have been described, and others can readily be identified, e.g., through sequence alignment, structural comparison, and similar techniques, as is well known in the art. Common sequence repositories for known proteins include GenBank and Swiss-Prot, and other repositories can easily be identified by searching the internet. Similarly, antibodies against phosphotyrosine, phosphoserine, and/or phosphothreonine are well known in the art; many are commercially available, and others can be generated by established techniques. Other domains suitable for use as detection modules include, e.g., death domains, PDZ domains, and SH3 domains. The detection module is optionally a polypeptide (e.g., a recombinant polypeptide, e.g., based on fibronectin) selected for binding to the first or second state of the substrate by a technique such as phage display, mRNA display, or another in vitro or in vivo display and/or selection technique.

[0051] A large number of kinases and kinase substrates have been described in the art and can be adapted to the practice of the present invention. For example, the enzyme can be chosen from any of sub-subclasses EC 2.7.10 - 2.7.12. In one class of embodiments, the kinase is a soluble (non-receptor) tyrosine kinase (for example, Abl, Arg, Blk, Bmx, Brk, BTK, Crk, Csk, DYRK1A, FAK, Fer, Fes/Fps, Fgr, Fyn, Hck, Itk, JAK, Lck, Lyn, MINK, Pyk, Src, Syk, Tec, Tyk, Yes, or ZAP-70), a receptor tyrosine kinase (for example, KIT, MET, KDR, EGFR, or an Eph receptor tyrosine kinase such as EphA1, EphA2, EphA3,

EphA4, EphA5, EphA7, EphB1, EphB3, EphB4, or EphB6), a member of a MAP kinase pathway (for example, ARAF1, BRAF1, GRB2, MAPK1, MAP2K1, RASA1, SOS1, MAP2K2, and MAPK3; see, e.g., Cobb et al. (1996) Promega Notes Magazine 59:37-41), a member of an Akt signal pathway (e.g., PTEN, CDKN1A, GSK3B, PDPK1, CDKN1B, ILK, AKT1, PIK3CA, and CCND1), or a member of an EGFR signal pathway (e.g., EGFR, ARAF1, BRAF1, GRB2, MAPK1, MAP2K1, RASA1, SOS1, and MAP2K2). Exemplary kinases include, but are not limited to, Src; AMP-K, AMP-activated protein kinase; βARK, β adrenergic receptor kinase; CaMK, CaM-kinase, calmodulin-dependent protein kinase; cdc2 kinase, protein kinase expressed by CDC2 gene; cdk, cyclin dependent kinase; CK1, protein kinase CK1 (also termed casein kinase 1 or I); CK2, protein kinase CK2 (also termed casein kinase 2 or II); CSK, C-terminal Src protein kinase; GSK3, glycogen synthase kinase-3; HCR, heme controlled repressor, HRI; HMG-CoA reductase kinase A; insulin receptor kinase; MAP kinase, ERK, extracellular signal-regulated kinase; MAP kinase activated protein kinase 1; MAP kinase activated protein kinase 2; MLCK, myosin light chain kinase; Nek, NIMA-related kinase; NIMA, never in mitosis protein kinase; p70 s6k and p90 srk, 70 and 90 kDa kinases that phosphorylate s6 protein; PDHK, pyruvate dehydrogenase kinase; PhK, phosphorylase kinase; PKA, cAMP-dependent protein kinase A; PKB, protein kinase B; PKG, cGMP-dependent protein kinase, protein kinase G; PKR, RNA-dependent protein kinase, dSRNA-PK; PKC, protein kinase C; PRK1, protein kinase C-related kinase 1; RAC; RhK, rhodopsin kinase; SNF-1 PK, sucrose non-fermenting protein kinase; Jun kinase, JNK; JNKKK; SrcN1, SrcN2, FynT, LynA, LynB, FGFR, TrkA, Flt3, and RSK.

[0052] Substrates for such kinases, including, e.g., protein substrates (e.g., another kinase, a histone, or myelin basic protein), amino acid polymers of random sequence (e.g., poly Glu/Tyr {4:1}), and/or polypeptide substrates with a defined amino acid sequence (e.g., chemically synthesized polypeptides; polypeptides including less than about 32 residues, less than about 20 residues, or less than about 15 residues; and polypeptides including between 7 and 15 residues), have been described in the art or can readily be determined by techniques known in art. See, e.g., Pinna and Ruzzene (1996) "How do protein kinases recognize their substrates?" Biochim Biophys Acta 1314:191-225. See, e.g., U.S. patent application 11/366,221 for a list of exemplary kinases and polypeptide substrates.

[0053] In another class of embodiments, the enzyme is a protein phosphatase. In this class of embodiments, the substrate in the first state is phosphorylated, and the substrate in the second state is unphosphorylated. In some embodiments, the detection module binds to

the substrate module when the substrate is in the second state; in other embodiments, the detection module binds to the substrate module when the substrate is in the first state (ie, the detection module binds to the phosphorylated substrate). Exemplary detection modules for the latter embodiments include those outlined above, e.g., SH2, PTB, 14-3-3, and other phosphoprotein binding domains, as well as antibodies and aptamers.

[0054] The phosphatase can be, e.g., a tyrosine-specific protein phosphatase (see, e.g., Alonso et al. (2004) "Protein Tyrosine Phosphatases in the Human Genome" Cell 117:699–711) or a serine/threonine-specific protein phosphatase (e.g., PP1, PP2A, PP2B, or PP2C). See also U.S. patent application 11/366,221. It will be evident that a phosphorylated kinase sensor (for example, phosphorylated versions of the exemplary kinase sensors described herein) can serve as a phosphatase sensor (and vice versa).

[0055] In another class of embodiments, the enzyme is a protein methyltransferase. For example, the enzyme can be a histone methyltransferase (e.g., a histone lysine methyltransferase or a histone arginine methyltransferase) or a protein lysine methyltransferase. In this class of embodiments, the substrate in the first state is unmethylated, and the substrate in the second state is methylated. The detection module is optionally, e.g., a polypeptide, an aptamer, or the like that recognizes the methylated substrate. For example, the detection module can include a chromodomain that binds a substrate including a methyllysine, a tudor domain that binds a substrate including a methylarginine, or an antibody. The substrate and detection modules optionally comprise distinct polypeptides.

[0056] In yet another class of embodiments, the enzyme is a protein acetyltransferase. For example, the enzyme can be a histone acetyltransferase or a lysine acetyltransferase. In this class of embodiments, the substrate in the first state is unacetylated, and the substrate in the second state is acetylated. The detection module is optionally, e.g., a polypeptide, an aptamer, or the like that recognizes the acetylated substrate. For example, the detection module can include a bromodomain that binds a substrate including an acetyllysine, or an antibody. The substrate and detection modules optionally comprise distinct polypeptides.

[0057] Methyltransferases, acetyltransferases, bromodomains and chromodomains have been described in the art. See, e.g., Yang (2004) "Lysine acetylation and the bromodomain: a new partnership for signaling" Bio.Essays 26:1076-1087, Berger (2002) "Histone modifications in transcriptional regulation" Curr. Opin. Genet. Dev. 12:142-148, Peterson and Laniel (2004) "Histones and histone modifications" Curr. Biol. 14:R546-R551, and Daniel et al. (2005) "Effector proteins for methylated histones" Cell Cycle 4:919-926.

[0058] A variety of fluorescent labels are known in the art and can be adapted to the practice of the present invention. In one aspect, the label is pyrene or a coumarin derivative. Further details can be found in the section entitled "Fluorescent labels" below.

[0059] The increase in signal from the fluorescent label upon binding of the substrate and detection modules can be substantial. For example, the increased intensity of fluorescent emission from the label is optionally an increase of at least about 7 fold, at least about 10 fold, at least about 20 fold, at least about 50 fold, at least about 60 fold, at least about 100 fold, or at least about 200 fold.

[0060] The substrate module optionally comprises a polypeptide comprising a Dap, Dab, ornithine, lysine, cysteine, or homocysteine residue (or essentially any other chemically reactive natural or unnatural amino acid derivative or residue) to which the fluorescent label is attached. The label can be attached to the residue (e.g., before or after its incorporation into a polypeptide) by reacting a derivative of the label with a functional group on the residue's side chain, for example. The label can be similarly attached to a free N-terminal amine on the polypeptide by reacting a derivative of the label with the amine, or the label can be introduced by incorporating a phorphoramidite including the label during chemical synthesis of the polypeptide, for example.

[0061] A variety of quenchers are known in the art and can be adapted to the practice of the present invention. See, for example, quenchers **D1-D48** in **Table S1** below. In one class of embodiments, the quencher is selected from the group consisting of Evans Blue, Reactive Blue, Eriochrome Black T, Alizarin Red, Aniline Blue WS, Chlorazol Black, Ponceau S, Rose Bengal, Tartrazine, Trypan Blue, and Acid Green 27. The quencher can be, e.g., an acceptor fluorophore, or it can be a dark quencher. In embodiments in which the quencher is a fluorophore, it is preferably a different fluorophore from the fluorescent label. The quencher is typically non-polymeric and is typically a small molecule (e.g., having a molecular weight of less than 1000 daltons, e.g., less than 500 daltons).

[0062] Preferably, when the substrate module is not bound to the detection module, the label exhibits little or no fluorescence. Thus, in one aspect, when the substrate module is not bound to the detection module, the quencher quenches fluorescent emission by the label by at least about 40%, as compared to fluorescent emission in the absence of the quencher. For example, the quencher can quench fluorescent emission by the label by at least about 50%, at least about 75%, at least about 90%, or at least about 95%, or can even prevent detectable emission from the label, e.g., at a given wavelength.

[0063] The quencher can quench fluorescent emission from the label when the label and quencher are in proximity, e.g., in solution. In one aspect, the quencher forms a non-covalent complex with the substrate module, putting the quencher in proximity to the label. The complex is stabilized by non-covalent interactions between the quencher and the label and/or substrate; for example, by electrostatic interactions, hydrophobic interactions, and/or hydrogen bonds between the quencher and the label and/or substrate (e.g., by electrostatic interactions between a negatively charged moiety on the quencher and positively charged side chain(s) on a polypeptide substrate and/or by hydrophobic interactions between the quencher and the label). Binding of the detection module to the substrate module disrupts the interactions between the quencher and the substrate module, disrupting the complex between the quencher and the substrate module and thereby and increasing the intensity of fluorescent emission from the label. In one class of embodiments, the non-covalent complex between the quencher and the substrate module has an apparent dissociation constant (apparent K_d) of about 20 μM or less, e.g., about 10 μM or less or even about 1 μM or less.

[0064] The molar ratio of the quencher to the substrate module in the composition can be varied, e.g., to achieve a desired level of quenching in the absence of binding of the substrate module to the detection module. For example, the molar ratio of the quencher to the substrate module in the composition can be at least about 1 to 1, at least about 5 to 1, at least about 10 to 1, at least about 25 to 1, or at least about 50 to 1.

[0065] The molar ratio of the detection module to the substrate module in the composition is optionally about 1 to 1. Typically, however, the detection module is present in excess (e.g., slight excess) relative to the substrate module. Thus, the molar ratio of the detection module to the substrate module in the composition is optionally greater than 1 to 1; for example, the molar ratio of the detection module to the substrate module can be at least about 2 to 1, at least about 5 to 1, or at least about 10 to 1.

[0066] The sensors can be used in biochemical assays of enzyme activity. Thus, the composition optionally includes the enzyme (e.g., a purified or partially purified enzyme), a cell or tissue lysate (e.g., a lysate including the enzyme), or a cell.

[0067] In one class of embodiments, the sensor is caged such that the enzyme can not act upon the substrate until the sensor is uncaged, for example, by removal of a photolabile caging group. Thus, in one class of embodiments, the sensor comprises one or more caging groups associated with the substrate module (e.g., with the substrate). The caging groups inhibit the enzyme from acting upon the substrate, e.g., by at least about 75%, at least about 90%, at least about 95%, or at least about 98%, as compared to the substrate in the absence of

the one or more caging groups. Preferably, the one or more caging groups prevent the enzyme from acting upon the substrate. Typically, removal of, or an induced conformational change in, the one or more caging groups permits the enzyme to act upon the substrate. The one or more caging groups associated with the substrate module can be covalently or non-covalently attached to the substrate module. In a preferred aspect, the one or more caging groups are photoactivatable (e.g., photolabile). For example, in one embodiment, the sensor comprises one or more photolabile caging groups covalently bound to the substrate, which caging groups inhibit or prevent the enzyme from acting upon the substrate. Caging groups are described in greater detail below, in the section entitled "Caging groups."

[0068] Caging of the sensor permits initiation of the reaction between the enzyme and the substrate within the sensor to be controlled, temporally and/or spatially. Similar or additional control of the reaction can be obtained through use of other caged reagents, for example, caged nucleotides (e.g., caged ATP), caged metal ions, caged chelating agents (e.g., caged EDTA or EGTA), caged activators or inhibitors, and the like. See, e.g., U.S. patent application publication 2004/0166553 by Nguyen et al. entitled "Caged sensors, regulators and compounds and uses thereof."

[0069] The sensor can be used to study the effects of activators and inhibitors (known and potential) on the enzyme's activity. Thus, the composition optionally includes a modulator or potential modulator of the activity of the enzyme.

[0070] Two or more enzyme activities can be monitored simultaneously or sequentially, if desired, by including in the composition a second sensor. The second sensor can, for example, comprise a second substrate module including a second substrate for a second enzyme and a second fluorescent label, whose signal is detectably different from that of the first sensor's label, and a second detection module. A second quencher is optionally also included, or, preferably, the same type of quencher quenches both labels. The second detection module can be the same as or different from the first detection module.

[0071] Other embodiments provide compositions including components of enzyme sensors (e.g., substrate and/or detection modules and/or quenchers) and/or nucleic acids encoding such components.

METHODS FOR DETECTING ENZYME ACTIVITY

[0072] In one aspect, the invention provides methods for assaying enzyme activity using sensors of the invention. Thus, one general class of embodiments provides methods of assaying an activity of an enzyme In the methods, the enzyme is contacted with a sensor. The sensor includes 1) a substrate module that comprises a substrate for the enzyme, wherein

the substrate is in a first state on which the enzyme can act, thereby converting the substrate to a second state, and a fluorescent label, 2) a detection module, which detection module binds to the substrate module when the substrate is in the first state, or which detection module binds to the substrate module when the substrate is in the second state, and 3) a quencher. In one aspect, the quencher is not covalently bound to the substrate module or to the detection module. Binding of the detection module to the substrate module results in an increased intensity of fluorescent emission from the label. The increased signal from the label is detected and correlated to the activity of the enzyme, thereby assaying the activity of the enzyme.

[0073] The assay can be, e.g., qualitative or quantitative. As a few examples, the assay can simply indicate whether the activity is present (e.g., an increase in intensity is detected) or absent (e.g., no signal change is detected), or it can indicate the activity is higher or lower than activity in a corresponding control sample (e.g., the increase in intensity is greater or less than that in a control assay or sample, e.g., one that includes a known quantity of enzyme or premodified substrate or the like), or it can be used to determine a number of activity units of the enzyme (an activity unit is typically defined as the amount of enzyme which will catalyze the transformation of 1 micromole of the substrate per minute under standard conditions).

[0074] The methods are optionally used, e.g., for in vitro biochemical assays of enzyme activity using purified or partially purified enzyme, a cell lysate, or the like. As noted previously, caging the sensor can permit initiation of the activity assay to be precisely controlled, temporally and/or spatially (see, e.g., U.S. patent application publication 2004/0166553). Thus, in one class of embodiments, the sensor comprises one or more caging groups associated with the substrate module (e.g., the substrate), which caging groups inhibit (e.g., prevent) the enzyme from acting upon the substrate. The methods include uncaging the substrate, e.g., by exposing the substrate to uncaging energy, thereby freeing the substrate from inhibition by the one or more caging groups. Typically, the one or more caging groups prevent the enzyme from acting upon the substrate, and removal of or an induced conformational change in the one or more caging groups permits the enzyme to act upon the substrate. The substrate can be uncaged, for example, by exposing the substrate to light of a first wavelength (for photoactivatable or photolabile caging groups), sonicating the substrate module, or otherwise supplying uncaging energy appropriate for the specific caging groups utilized.

[0075] Alternatively or in addition, the methods can include uncaging other caged reagents, for example, caged nucleotides (e.g., caged ATP, e.g., to initiate a kinase reaction),

caged metal ions, caged chelating agents (e.g., caged EDTA or EGTA, e.g., to terminate a reaction requiring divalent cations), caged activators or inhibitors, or the like.

[0076] The methods can include contacting the enzyme with a modulator (e.g., an activator or inhibitor) of its activity. Similarly, the methods can include modulating the activity of at least one other enzyme, e.g., by adding an activator or inhibitor of at least one other enzyme that functions (or potentially functions) in an upstream, downstream, or related signaling or metabolic pathway.

[0077] In one aspect, the methods can be used to screen for compounds that affect activity of the enzyme (or binding of the substrate and detection modules to each other). Thus, in one class of embodiments, the methods include contacting the enzyme with a test compound, assaying the activity of the enzyme in the presence of the test compound, and comparing the activity of the enzyme in the presence of the test compound with the activity of the enzyme in the absence of the test compound.

[0078] The methods can be used to monitor the activities of two or more enzymes, e.g., in a single reaction mixture. For example, if desired, a second sensor comprising a second substrate module including a second substrate for a second enzyme, a second fluorescent label whose signal is detectably different from that of the first sensor's label, a second detection module, and optionally a second quencher, is contacted with the second enzyme. The second detection module and/or quencher can be the same as or different from the first detection module and/or quencher. An increase in signal from the second label is detected and correlated with the activity of the second enzyme.

[0079] Essentially all of the features noted for the compositions above apply to these methods as well, as relevant: for example, with respect to type of enzyme, exemplary substrate and/or detection modules, type of fluorescent label and/or quencher, degree of quenching, fold increase in fluorescence emission, molar ratio of the substrate module to the quencher and/or the detection module, type of caging groups, and/or the like. For example, the quencher can form a non-covalent complex with the substrate module. Binding of the substrate and detection modules disrupts the complex between the quencher and the substrate module, thereby increasing the intensity of fluorescent emission from the label. As for the embodiments above, the non-covalent complex between the quencher and the substrate module optionally has an apparent K_d of about 20 μM or less, e.g., about 10 μM or less or even about 1 μM or less.

BINDING SENSORS

[0080] One aspect of the invention provides binding sensors (e.g., combinations of labeled polypeptides and quenchers) for detecting or monitoring an intermolecular association, e.g., between two polypeptides. Accordingly, one general class of embodiments provides a composition including a labeled polypeptide comprising a first polypeptide and a fluorescent label, a second polypeptide to which the first polypeptide binds, and a quencher. Binding of the first polypeptide to the second polypeptide results in an increased intensity of fluorescent emission from the label, since the label is at least partially sequestered from the quencher In one aspect, the quencher is not covalently bound to the first polypeptide or to the second polypeptide.

[0081] A wide variety of domains known to recognize various amino acid sequences have been described in the art and can be employed as first or second polypeptides. See, for example, the references above and pawsonlab (dot) mshri (dot) on (dot) ca/index (dot) php?option=com_content&task=view&id=30&Itemid=63. Exemplary domains useful as or in second polypeptides include, but are not limited to, LIM, PDZ, WW, FHA, SH3, 14-3-3, SH2, PTB, chromo-, and bromo- domains.

[0082] In one exemplary class of embodiments, the first polypeptide is a proline rich polypeptide and the second polypeptide comprises an SH3 domain. In another class of embodiments, the first polypeptide comprises a phosphorylated serine residue and the second polypeptide comprises a 14-3-3 domain. In yet another class of embodiments, the first polypeptide comprises a phosphorylated tyrosine residue and the second polypeptide comprises an SH2 or PTB domain. In yet another class of embodiments, the first polypeptide comprises a methylated lysine residue and the second polypeptide comprises a chromodomain, or the first polypeptide comprises an acetylated lysine residue and the second polypeptide comprises a bromodomain.

[0083] It will be evident that the substrate modules (or modified forms thereof) and/or detection modules described for the enzyme sensors above can be adapted for use as first and/or second polypeptides in these embodiments. Thus, in one exemplary class of embodiments, the first polypeptide comprises amino acid sequence $X^{-4}\,R^{-3}\,R^{-2}\,X^{-1}\,S^{0}\,X^{+1}\,X^{+2}$ (SEQ ID NO:13), wherein S^0 is phosphorylated; where X^{-4} and X^{+2} are independently selected from the group consisting of: an amino acid residue and an amino acid residue comprising the fluorescent label; and where X^{-1} and X^{+1} are independently selected from the group consisting of: a hydrophobic amino acid residue and an amino acid residue comprising the fluorescent label. For example, the labeled polypeptide can be any one of **P1-P12** (SEQ

ID NOs:1-12) in which the serine residue is phosphorylated, and the second polypeptide optionally comprises a 14-3-3 domain. As a few specific examples, the labeled polypeptide can be serine-phosphorylated **P5** and the quencher Rose Bengal, the labeled polypeptide serine-phosphorylated **P9** and the quencher Aniline Blue WS, the labeled polypeptide serine-phosphorylated **P2** and the quencher Ponceau S, or the labeled polypeptide serine-phosphorylated **P12** and the quencher Acid Green 27.

[0084] Essentially all of the features noted for the embodiments above apply to these embodiments as well, as relevant: for example, with respect to type of fluorescent label, type of quencher, and/or the like.

[0085] For example, it is worth noting that the binding sensors are optionally caged In one class of embodiments, the sensor is caged such that the first and second polypeptides can not bind to each other until the sensor is uncaged, for example, by removal of a photolabile caging group. Thus, in one class of embodiments, the labeled polypeptide comprises one or more caging groups associated with the first polypeptide. The caging groups inhibit the first polypeptide from binding to the second polypeptide, e.g., by at least about 75%, at least about 90%, at least about 95%, or at least about 98%, as compared to binding in the absence of the one or more caging groups. Preferably, the one or more caging groups prevent the first polypeptide from binding to the second polypeptide. Typically, removal of, or an induced conformational change in, the one or more caging groups permits the first polypeptide to bind to the second polypeptide. The one or more caging groups associated with the first polypeptide can be covalently or non-covalently attached to polypeptide. In a preferred aspect, the one or more caging groups are photoactivatable (e.g., photolabile). For example, in one embodiment, the labeled polypeptide comprises one or more photolabile caging groups covalently bound to the first polypeptide, which caging groups inhibit or prevent the first polypeptide from binding to the second polypeptide. As noted above, caging groups are described in greater detail below, in the section entitled "Caging groups."

[0086] The increase in signal from the fluorescent label upon binding of the first and second polypeptides is optionally an increase of at least about 7 fold, at least about 10 fold, at least about 20 fold, at least about 50 fold, at least about 60 fold, at least about 100 fold, or at least about 200 fold.

[0087] Preferably, when the labeled first polypeptide is not bound to the second polypeptide, the label exhibits little or no fluorescence. Thus, in one aspect, when the first polypeptide is not bound to the second polypeptide, the quencher quenches fluorescent emission by the label by at least about 40%, as compared to fluorescent emission in the

absence of the quencher. For example, the quencher can quench fluorescent emission by the label by at least about 50%, at least about 75%, at least about 90%, or at least about 95%, or can even prevent detectable emission from the label, e.g., at a given wavelength.

[0088] The quencher can quench fluorescent emission from the label when the label and quencher are in proximity, e.g., in solution. In one aspect, the quencher forms a non-covalent complex with the labeled polypeptide, putting the quencher in proximity to the label. The complex is stabilized by non-covalent interactions between the quencher and the label and/or first polypeptide, for example, by electrostatic interactions, hydrophobic interactions, and/or hydrogen bonds between the quencher and the label and/or first polypeptide (e.g., by electrostatic interactions between a negatively charged moiety on the quencher and positively charged side chain(s) on the first polypeptide and/or by hydrophobic interactions between the quencher and the label). Binding of the second polypeptide to the labeled polypeptide disrupts the interactions between the quencher and the labeled polypeptide, disrupting the complex between the quencher and the labeled polypeptide and thereby increasing the intensity of fluorescent emission from the label. In one class of embodiments, the non-covalent complex between the quencher and the labeled polypeptide has an apparent dissociation constant (apparent K_d) of about 20 μM or less, e.g., about 10 μM or less or even about 1 μM or less.

[0089] The molar ratio of the quencher to the labeled polypeptide in the composition can be varied, e.g., to achieve a desired level of quenching in the absence of binding of the first polypeptide to the second polypeptide. For example, the molar ratio of the quencher to the labeled polypeptide in the composition can be at least about 1 to 1, at least about 5 to 1, at least about 10 to 1, at least about 25 to 1, or at least about 50 to 1.

[0090] The binding sensors can be used to study the effects of compounds that affect (potentiate or inhibit) or potentially affect the interaction between the first and second polypeptides. Thus, the composition optionally includes an inhibitor or potential inhibitor of the interaction between the first and second polypeptides, for example, a compound that competes with the first polypeptide for binding to the second polypeptide or a compound that noncompetitively inhibits binding of the first polypeptide to the second polypeptide.

[0091] A second binding sensor (e.g., including a second, detectably different label) is optionally included in the composition to monitor an additional protein-protein interaction. Other embodiments provide compositions including components of the binding sensor compositions (e.g., first polypeptides, quenchers, and/or second polypeptides) and/or nucleic acids encoding such components.

METHODS FOR ASSAYING PROTEIN-PROTEIN INTERACTIONS

[0092] One general class of embodiments provides methods of assaying an intermolecular interaction between a first polypeptide and a second polypeptide. In the methods, a labeled polypeptide comprising the first polypeptide and a fluorescent label is provided, as is a quencher. In one aspect, the quencher is not covalently bound to the first polypeptide or to the second polypeptide. The labeled polypeptide, the quencher, and the second polypeptide are contacted, thereby permitting the first polypeptide to bind to the second polypeptide. Binding of the first polypeptide to the second polypeptide results in an increased intensity of fluorescent emission from the label. The increased intensity of fluorescent emission is detected and correlated to binding of the first and second polypeptides.

[0093] The assay can be, e.g., qualitative or quantitative. As a few examples, the assay can simply indicate whether the protein-protein interaction occurs (e.g., an increase in intensity is detected) or does not occur (e.g., no signal change is detected), or it can indicate the extent to which the interaction occurs as compared to a corresponding control sample (e.g., the increase in intensity is greater or less than that in a control assay or sample, e.g., one that includes a known quantity of second polypeptide), or it can be used to quantitate the interaction in some way (e.g., to determine a K_d for the protein-protein complex).

[0094] The methods are optionally used, e.g., for in vitro biochemical assays of intermolecular interactions using purified or partially purified enzyme, a cell lysate, or the like. As for the embodiments above, caging the binding sensor can permit initiation of the assay to be precisely controlled, temporally and/or spatially. Thus, in one class of embodiments, the labeled polypeptide comprises one or more caging groups associated with the first polypeptide, which caging groups inhibit (e.g., prevent) the first polypeptide from binding to the second polypeptide. The methods include uncaging the first polypeptide, e.g., by exposing the first polypeptide to uncaging energy, thereby freeing the first polypeptide from inhibition by the one or more caging groups. Typically, the one or more caging groups prevent the first polypeptide from binding to the second polypeptide, and removal of or an induced conformational change in the one or more caging groups permits the first polypeptide to bind to the second polypeptide. The first polypeptide can be uncaged, for example, by exposing it to light of a first wavelength (for photoactivatable or photolabile caging groups), sonicating it, or otherwise supplying uncaging energy appropriate for the specific caging groups utilized.

[0095] The methods can be used to monitor the interaction of two or more sets of molecules, e.g., in a single reaction mixture, by using a second binding sensor. The methods can include contacting the enzyme with a compound that affects (potentiates or inhibits) or potentially affects the interaction between the first and second polypeptides.

[0096] In one aspect, the methods can be used to screen for compounds (e.g., synthetic peptides, small molecules, etc) that affect the interaction between the first and second polypeptides. Thus, in one class of embodiments, the methods include contacting the second polypeptide with a test compound, assaying the interaction between the first and second polypeptides in the presence of the test compound, and comparing the interaction between the first and second polypeptides in the presence of the test compound with interaction between the first and second polypeptides in the absence of the test compound. The test compound is optionally one that inhibits binding of the first and second polypeptides, for example, a compound that competes with the first polypeptide for binding to the second polypeptide. For example, the test compound is optionally a compound (e.g., a synthetic peptide) that binds to a 14-3-3, SH2, SH3, PTB, chromo-, or bromo- domain.

[0097] As just one example, the methods can be used in a screen to identify inhibitory ligands for 14-3-3 proteins. High fluorescence is observed when a suitable labeled polypeptide and a quencher (e.g., one of the combinations described herein, such as serine-phosphorylated **P5** and Rose Bengal, serine-phosphorylated **P9** and Aniline Blue WS, serine-phosphorylated **P2** and Ponceau S, or serine-phosphorylated **P12** and Acid Green 27) are contacted with a second polypeptide including a 14-3-3 domain. Screening through a library of potential 14-3-3 inhibitory ligands can be conducted simply by contacting each member of the library (singly or in combination) with the labeled polypeptide, quencher, and second polypeptide; promising compounds (inhibitory ligands) generate a drop in fluorescent intensity, typically, a substantial decrease in or even elimination of observed fluorescence. Such inhibitors are of interest, for example, as therapeutic agents to block signaling through 14-3-3-mediated pathways involved in diseases such as cancer. See, e.g., Wilker and Yaffe (2004) "14-3-3 proteins - a focus on cancer and human disease" J. Mol. Cell Cardiol. 37:633-642.

[0098] Essentially all of the features noted for the embodiments above apply to these methods as well, as relevant: for example, with respect to exemplary first and/or second polypeptides, type of fluorescent label and/or quencher, degree of quenching, fold increase in fluorescence emission, molar ratio of the labeled polypeptide to the quencher and/or the second polypeptide, and/or the like. For example, the quencher can form a non-covalent

complex with the labeled polypeptide. Binding of the first and second polypeptides disrupts the complex between the quencher and the labeled polypeptide, thereby increasing the intensity of fluorescent emission from the label. As for the embodiments above, the non-covalent complex between the quencher and the labeled polypeptide optionally has an apparent K_d of about 20 μM or less, e.g., about 10 μM or less or even about 1 μM or less.

KITS

[0099] Kits comprising components of compositions of the invention and/or that can be used in practicing the methods of the invention form another feature of the invention. For example, in one class of embodiments, the kit includes a sensor for detecting an activity of an enzyme, packaged in one or more containers. The sensor comprises a substrate module, a detection module, and a quencher. The substrate module includes a substrate for the enzyme, wherein the substrate is in a first state on which the enzyme can act, thereby converting the substrate to a second state, and a fluorescent label. The detection module binds to the substrate module when the substrate is in the first state or when the substrate is in the second state. Binding of the detection module to the substrate module results in an increased intensity of fluorescent emission from the label, since the label is at least partially sequestered from the quencher. In one aspect, the quencher is not covalently bound to the substrate module or to the detection module. Typically, the kit also includes instructions for using the sensor to detect the activity of the enzyme. The kit optionally also includes one or more buffers, controls including a known quantity of the enzyme, and/or the like. Essentially all of the features noted for the compositions above apply to these kits as well, as relevant: for example, with respect to type of enzyme, exemplary substrate and/or detection modules, type of fluorescent label and/or quencher, inclusion of caging groups, and/or the like.

[0100] In another class of embodiments, a kit includes a sensor for detecting or monitoring an intermolecular association, e.g., between two polypeptides. The kit includes a quencher and a labeled polypeptide comprising a first polypeptide and a fluorescent label, packaged in one or more containers. The first polypeptide is capable of binding to a second polypeptide, where binding of the first polypeptide to the second polypeptide results in an increased intensity of fluorescent emission from the label, since the label is at least partially sequestered from the quencher. In one aspect, the quencher is not covalently bound to the first polypeptide or to the second polypeptide. Typically, the kit also includes instructions for using the sensor to assay the protein-protein interaction. The kit optionally also includes one or more buffers, controls including a known quantity of the second polypeptide, and/or the like. Essentially all of the features noted for the compositions above apply to these kits as

well, as relevant: for example, with respect to exemplary first and/or second polypeptides, type of fluorescent label and/or quencher, inclusion of caging groups, and/or the like.

SYSTEMS

[0101] In one aspect, the invention includes systems, e.g., systems used to practice the methods herein and/or comprising the compositions described herein. The system can include, e.g., a fluid handling element, a fluid containing element, a laser for exciting a fluorescent label, a detector for detecting a signal from a label (e.g., fluorescent emissions from a fluorescent label), a source of uncaging energy for uncaging caged sensors, and/or a robotic element that moves other components of the system from place to place as needed (e.g., a multiwell plate handling element). For example, in one class of embodiments, a composition of the invention is contained in a microplate reader or like instrument.

[0102] The system can optionally include a computer. The computer can include appropriate software for receiving user instructions, either in the form of user input into a set of parameter fields, e.g., in a GUI, or in the form of preprogrammed instructions, e.g., preprogrammed for a variety of different specific operations. The software optionally converts these instructions to appropriate language for controlling the operation of components of the system (e.g., for controlling a fluid handling element, robotic element, and/or laser). The computer can also receive data from other components of the system, e.g., from a detector, and can interpret the data (e.g., by correlating a change in signal from the label with an activity of an enzyme or with a protein-protein interaction), provide it to a user in a human readable format, or use that data to initiate further operations, in accordance with any programming by the user.

FLUORESCENT LABELS

[0103] As noted, the various sensors and labeled polypeptides of this invention include fluorescent labels. A wide variety of fluorescent labels have been described in the art and can be adapted to the practice of the present invention. Examples include, but are not limited to, dapoxyl, NBD, Cascade Yellow, dansyl, PyMPO, pyrene, 7-diethylaminocoumarin-3-carboxylic acid, Marina Blue™, Pacific Blue™, Cascade Blue™, 2-anthracenesulfonyl, PyMPO, 3,4,9,10-perylene-tetracarboxylic acid, 2,7-difluorofluorescein (Oregon Green™ 488-X), 5-carboxyfluorescein, Texas Red™-X, Alexa Fluor 430, 5-carboxytetramethylrhodamine (5-TAMRA), 6-carboxytetramethylrhodamine (6-TAMRA), BODIPY FL, bimane, and Alexa Fluor 350, 405, 488, 500, 514, 532, 546, 555, 568, 594, 610, 633, 647, 660, 680, 700, and 750, and derivatives thereof, among many others. For example, various derivatives of coumarins are described in Section 1.7 of "The Handbook - A Guide to

Fluorescent Probes and Labeling Technologies, Tenth Edition," available on the internet at probes (dot) invitrogen (dot) com/handbook. Fluorescent labels employed in the invention are optionally small molecules, e.g., having a molecular weight of less than about 1000 daltons.

[0104] The labels are optionally environmentally sensitive or environmentally insensitive labels. Environmentally insensitive labels are preferred in certain embodiments, since such labels typically provide brighter emissions. The fluorescence of an environmentally insensitive fluorescent label is typically not significantly affected by the solvent in which the label is located. For example, the signal from an environmentally insensitive fluorescent label is typically not significantly different whether the label is in an aqueous solution, a less polar solvent (e.g., methanol),or a nonpolar solvent (e.g., hexane). In contrast, the signal from an environmentally sensitive label changes when the environment of the label changes. For example, the fluorescence of an environmentally sensitive fluorescent label changes when the hydrophobicity, pH, and/or the like of the label's environment changes (e.g., upon binding of the substrate module with which the label is associated to a detection module, such that the label is transferred from an aqueous environment to a more hydrophobic environment at the binding interface between the modules). Typically, the signal from an environmentally sensitive label is affected by the solvent in which the label is located. For example, the signal from an environmentally sensitive fluorescent label is typically significantly different when the label is in an aqueous solution versus in a less polar solvent (e.g., methanol) versus in a nonpolar solvent (e.g., hexane). Examples of environmentally sensitive fluorophores include, but are not limited to, those described in U.S. patent application 11/366,221 and references therein, including in U.S. patent application publication 2002/0055133 by Hahn et al. entitled "Labeled peptides, proteins and antibodies and processes and intermediates useful for their preparation."

[0105] Signals from the fluorescent labels can be detected by essentially any method known in the art (e.g., fluorescence spectroscopy, fluorescence microscopy, etc.). Excitation and emission wavelengths for the exemplary fluorophores described above can be found, e.g., in "The Handbook - A Guide to Fluorescent Probes and Labeling Technologies, Tenth Edition," available on the internet at probes (dot) invitrogen (dot) com/handbook, and in the references above.

[0106] Labels can be attached to molecules (e.g., substrates) during synthesis or by postsynthetic reactions by techniques established in the art. For example, a fluorescently labeled nucleotide can be incorporated into a nucleic acid during enzymatic or chemical synthesis of the nucleic acid, e.g., at a preselected or random nucleotide position.

Alternatively, fluorescent labels can be added to nucleic acids by postsynthetic reactions, at either random or preselected positions (e.g., an oligonucleotide can be chemically synthesized with a terminal amine or free thiol at a preselected position, and a fluorophore can be coupled to the oligonucleotide via reaction with the amine or thiol). Reactive forms of various fluorophores are commercially available e.g., from Molecular Probes, Inc, or can readily be prepared by one of skill in the art and used for incorporation of the labels into desired molecules. As another example, a fluorescently labeled residue can be incorporated into a polypeptide during enzymatic or chemical synthesis of the polypeptide. Alternatively, fluorescent labels can be added to polypeptides by postsynthetic reactions. A polypeptide substrate optionally comprises one or more residues incorporated to facilitate attachment of the label, e.g., an (L)-2,3-diaminopropionic acid (Dap), (L)-2,4-diaminobutyric acid (Dab), ornithine, lysine, cysteine, or homocysteine residue (or essentially any other chemically reactive natural or unnatural amino acid derivative or residue) to which the label is attached. See, e.g., the Examples sections herein, and U.S. patent application 11/366,221 and US patent application publication 2002/0055133.

CAGING GROUPS

[0107] A large number of caging groups, and a number of reactive compounds that can be used to covalently attach caging groups to other molecules, are well known in the art. Examples of photolabile caging groups include, but are not limited to: nitroindolines; N-acyl-7-nitroindolines; phenacyls; hydroxyphenacyl; brominated 7-hydroxycoumarin-4-ylmethyls (e.g., Bhc); benzoin esters; dimethoxybenzoin; meta-phenols; 2-nitrobenzyl; 1-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE); 4,5-dimethoxy-2-nitrobenzyl (DMNB); alpha-carboxy-2-nitrobenzyl (CNB); 1-(2-nitrophenyl)ethyl (NPE); 5-carboxymethoxy-2-nitrobenzyl (CMNB); (5-carboxymethoxy-2-nitrobenzyl)oxy) carbonyl; (4,5-dimethoxy-2-nitrobenzyl)oxy) carbonyl; desoxybenzoinyl; and the like. *See*, e.g., U.S. Patent No. 5,635,608 to Haugland and Gee (June 3, 1997) entitled "α-carboxy caged compounds"; Neuro 19, 465 (1997); J. Physiol. 508.3, 801 (1998); Proc. Natl. Acad. Sc.i USA 1988 Sep, 85(17):6571-5; J. Biol. Chem. 1997 Feb 14, 272(7):4172-8; Neuron 20, 619-624, 1998; Nature Genetics, vol 28:2001:317-325; Nature, vol 392,1998:936-941; Pan, P, and Bayley, H "Caged cysteine and thiophosphoryl peptides" FEBS Letters 405:81-85 (1997); Pettit et al (1997) "Chemical two-photon uncaging: a novel approach to mapping glutamate receptors" Neuron 19:465-471; Furuta et al. (1999) "Brominated 7-hydroxycoumarin-4-ylmethyls: novel photolabile protecting groups with biologically useful cross-sections for two photon

photolysis" Proc. Natl. Acad. Sci. 96(4):1193-1200; Zou et al. "Catalytic subunit of protein kinase A caged at the activating phosphothreonine" J. Amer. Chem. Soc. (2002) 124:8220-8229; Zou et al. "Caged Thiophosphotyrosine Peptides" Angew. Chem. Int. Ed. (2001) 4:3049-3051; Conrad II et al. "p-Hydroxyphenacyl Phototriggers: The reactive Excited State of Phosphate Photorelease" J. Am. Chem. Soc. (2000) 122:9346-9347; Conrad II et al. "New Phototriggers 10: Extending the π,π^* Absorption to Release Peptides in Biological Media" Org. Lett. (2000) 2:1545-1547; Givens et al. "A New Phototriggers 9: p-Hydroxyphenacyl as a C-Terminus Photoremovable Protecting Group for Oligopeptides" J. Am. Chem. Soc. (2000) 122:2687-2697; Bishop et al. "40-Aminomethyl-2,20-bipyridyl-4-carboxylic Acid (Abc) and Related Derivatives: Novel Bipyridine Amino Acids for the Solid-Phase Incorporation of a Metal Coordination Site Within a Peptide Backbone" Tetrahedron (2000) 56:4629-4638; Ching et al. "Polymers As Surface-Based Tethers with Photolytic triggers Enabling Laser-Induced Release/Desorption of Covalently Bound Molecules" Bioconjugate Chemistry (1996) 7:525-8; "BioProbes Handbook," 2002 from Molecular Probes, Inc; and "Handbook of Fluorescent Probes and Research Products," Ninth Edition or Web Edition, from Molecular Probes, Inc, as well as the references below. Many compounds, kits, etc for use in caging various molecules are commercially available, e.g., from Molecular Probes, Inc (www (dot) molecularprobes (dot) com).

[0108] Environmentally responsive polymers suitable for use as caging groups have also been described. Such polymers undergo conformational changes induced by light, an electric or magnetic field, a change in pH and/or ionic strength, temperature, or addition of an antigen or saccharide, or other environmental variables. For example, Shimoboji et al. (2002) "Photoresponsive polymer-enzyme switches" Proc. Natl. Acad. Sci. USA 99:16,592-16,596 describes polymers that undergo reversible conformational changes in response to light. Such polymers can, e.g., be used as photoactivatable caging groups. See U.S. patent application publication 2004/0166553. See also Ding et al. (2001) "Size-dependent control of the binding of biotinylated proteins to streptavidin using a polymer shield" Nature 411:59-62; Miyata et al. (1999) "A reversibly antigen-responsive hydrogel" Nature 399:766-769; Murthy et al. (2003) "Bioinspired pH-responsive polymers for the intracellular delivery of biomolecular drugs" Bioconjugate0 Chem. 14:412-419; and Galaev and Mattiasson (1999) "'Smart' polymers and what they could do in biotechnology and medicine" Trends Biotech. 17:335-340.

[0109] An alternative method for caging a molecule is to enclose the molecule in a photolabile vesicle (e.g., a photolabile lipid vesicle), optionally including a protein transduction domain or the like. Similarly, the molecule can be loaded into the pores of a porous bead which is then encased in a photolabile gel. As another alternative, a caging group optionally comprises a first binding moiety that can bind to a second binding moiety. For example, the caging group can include a biotin (the first binding moiety in this example); a second binding moiety, e.g., streptavidin or avidin, can thus be bound to the caging group, increasing its bulkiness and its effectiveness at caging. In certain embodiments, a caged component comprises two or more caging groups each comprising a first binding moiety, and the second binding moiety can bind two or more first binding moieties simultaneously. See U.S. patent application publication 2004/0166553.

[0110] Caged polypeptides (including, e.g., polypeptide substrates, substrate modules, and detection modules) can be produced, e.g., by reacting a polypeptide with a caging compound or by incorporating a caged amino acid during synthesis of a polypeptide. See, e.g., Tatsu et al. (1996) "Solid-phase synthesis of caged peptides using tyrosine modified with a photocleavable protecting group: Application to the synthesis of caged neuropeptide Y" Biochem. Biophys. Res. Comm. 227:688-693, which describes synthesis of polypeptides including tyrosine residues caged with 2-nitrobenzyl groups; Veldhuyzen et al. (2003) "A light-activated probe of intracellular protein kinase activity" J. Am. Chem. Soc. 125:13358-9, which describes synthesis of a polypeptide including a caged serine; and Vazquez et al. (2003) "Fluorescent caged phosphoserine peptides as probes to investigate phosphorylation-dependent protein associations" J. Am. Chem. Soc. 125:10150-10151, which describes synthesis of a polypeptide including a caged phosphoserine. See also, e.g., U.S. Patent No. 5,998,580 to Fay et al. (December 7, 1999) entitled "Photosensitive caged macromolecules"; Kossel et al. (2001) PNAS 98:14702-14707; Trends Plant Sci (1999) 4:330-334; PNAS (1998) 95:1568-1573; J. Am. Chem. Soc. (2002) 124:8220-8229; Pharmacology & Therapeutics (2001) 91:85-92; and Angew. Chem. Int. Ed. Engl. (2001) 4:3049-3051. A photolabile polypeptide linker can, for example, comprise a photolabile amino acid such as that described in U.S. Patent No. 5,998,580, *supra*.

[0111] Caged nucleic acids (e.g., DNA, RNA or PNA) can be produced by reacting the nucleic acids with caging compounds or by incorporating a caged nucleotide during synthesis of a nucleic acid. *See*, e.g., U.S. Patent No. 6,242,258 to Haselton and Alexander (June 5, 2001) entitled "Methods for the selective regulation of DNA and RNA transcription and

translation by photoactivation"; Nature Genetics (2001) 28: 317-325; and Nucleic Acids Res. (1998) 26:3173-3178.

[0112] Caged modulators (e.g., inhibitors and activators), small molecules, etc can be similarly produced by reaction with caging compounds or by synthesis. *See*, e.g., Trends Plant Sci. (1999) 4:330-334; PNAS (1998) 95:1568-1573; U.S. Patent No. 5,888,829 to Gee and Millard (March 30, 1999) entitled "Photolabile caged ionophores and method of using in a membrane separation process"; U.S. Patent No. 6,043,065 to Kao et al. (March 28, 2000) entitled "Photosensitive organic compounds that release 2,5,-di(tert-butyl) hydroquinone upon illumination"; U.S. Patent No. 5,430,175 to Hess et al. (July 4, 1995) entitled "Caged carboxyl compounds and use thereof"; U.S. Patent No. 5872243; and PNAS (1980) 77:7237-41. A number of caged compounds, including for example caged nucleotides, caged Ca2+, caged chelating agents, caged neurotransmitters, and caged luciferin, are commercially available, e.g., from Molecular Probes, Inc (on the world wide web at molecularprobes (dot) com).

[0113] Useful site(s) of attachment of caging groups to a given molecule can be determined by techniques known in the art. For example, a molecule with a known activity can be reacted with a caging compound. The resulting caged molecule can then be tested to determine if its activity is sufficiently abrogated. As another example, amino acid residues central to the activity of a polypeptide substrate (e.g., a residue modified by the enzyme, residues located at a binding interface, or the like) can be identified by routine techniques such as scanning mutagenesis, sequence comparisons and site-directed mutagenesis, or the like. Such residues can then be caged, and the activity of the caged substrate can be assayed to determine the efficacy of caging.

[0114] Appropriate methods for uncaging caged molecules are also known in the art. For example, appropriate wavelengths of light for removing many photolabile groups have been described; e.g., 300-360 nm for 2-nitrobenzyl, 350 nm for benzoin esters, and 740 nm for brominated 7-hydroxycoumarin-4-ylmethyls (two-photon) (see, e.g., references herein). Conditions for uncaging any caged molecule (e.g., the optimal wavelength for removing a photolabile caging group) can be determined according to methods well known in the art. Instrumentation and devices for delivering uncaging energy are likewise known (e.g., sonicators, heat sources, light sources, and the like). For example, well-known and useful light sources include e.g., a lamp, a laser (e.g., a laser optically coupled to a fiber-optic delivery system) or a light-emitting compound. See also U.S. patent application 10/716,176 by Witney et al. entitled "Uncaging devices."

MOLECULAR BIOLOGICAL TECHNIQUES

[0115] In practicing the present invention, many conventional techniques in molecular biology, microbiology, and recombinant DNA technology are optionally used (e.g., for making and/or manipulating nucleic acids, polypeptides, and/or cells of the invention). These techniques are well known, and detailed protocols for numerous such procedures (including, e.g., in vitro amplification of nucleic acids, cloning, mutagenesis, transformation, cellular transduction with nucleic acids, protein expression, and/or the like) are described in, for example, Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc, San Diego, CA; Sambrook et al., Molecular Cloning - A Laboratory Manual (3rd Ed), Vol 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 2002; and Current Protocols in Molecular Biology, FM Ausubel et al., eds, Current Protocols, a joint venture between Greene Publishing Associates, Inc and John Wiley & Sons, Inc, (supplemented through 2006)). Other useful references, e.g. for cell isolation and culture include Freshney (1994) Culture of Animal Cells, a Manual of Basic Technique, third edition, Wiley- Liss, New York and the references cited therein; Payne et al. (1992) Plant Cell and Tissue Culture in Liquid Systems John Wiley & Sons, Inc New York, NY; Gamborg and Phillips (Eds) (1995) Plant Cell, Tissue and Organ Culture; Fundamental Methods Springer Lab Manual, Springer-Verlag (Berlin Heidelberg New York) and Atlas and Parks (Eds) The Handbook of Microbiological Media (1993) CRC Press, Boca Raton, FL. A variety of vectors, including expression vectors, have been described and are readily available to one of skill, as are a large number of cells and cell lines suitable for the maintenance and use of such vectors.

POLYPEPTIDE PRODUCTION

[0116] Polypeptides (e.g., polypeptide substrates, detection modules, substrate modules, etc) can optionally be produced by expression in a host cell transformed with a vector comprising a nucleic acid encoding the desired polypeptide(s). Expressed polypeptides can be recovered and purified from recombinant cell cultures by any of a number of methods well known in the art, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography (e.g., using any of the tagging systems noted herein), hydroxylapatite chromatography, and lectin chromatography, for example. Protein refolding steps can be used, as desired, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed in the final purification steps See, e.g., the references noted above and Deutscher, "Methods in

Enzymology Vol 182: Guide to Protein Purification," Academic Press, Inc NY (1990); Sandana (1997) Bioseparation of Proteins, Academic Press, Inc; Bollag et al. (1996) "Protein Methods," 2nd Edition Wiley-Liss, NY; Walker (1996) "The Protein Protocols Handbook" Humana Press, NJ; Harris and Angal (1990) "Protein Purification Applications: A Practical Approach" IRL Press at Oxford, Oxford, UK; Scopes (1993) "Protein Purification: Principles and Practice" 3rd Edition Springer Verlag, NY; Janson and Ryden (1998) "Protein Purification: Principles, High Resolution Methods and Applications," Second Edition Wiley-VCH, NY; and Walker (1998) "Protein Protocols" on CD-ROM Humana Press, NJ.

[0117] Alternatively, cell-free transcription/translation systems can be employed to produce polypeptides encoded by nucleic acids. A number of suitable in vitro transcription and translation systems are commercially available. A general guide to in vitro transcription and translation protocols is found in Tymms (1995) "In vitro Transcription and Translation Protocols: Methods in Molecular Biology" Volume 37, Garland Publishing, NY.

[0118] In addition, polypeptides (including, e.g., polypeptides comprising fluorophores and/or unnatural amino acids) can be produced manually or by using an automated system, by direct peptide synthesis using solid-phase techniques (see, e.g., Chan and White, Eds, (2000) Fmoc Solid Phase Peptide Synthesis: A Practical Approach, Oxford University Press, New York, New York; Lloyd-Williams, P et al. (1997) Chemical Approaches to the Synthesis of Peptides and Proteins, CRC Press; Stewart et al. (1969) Solid-Phase Peptide Synthesis, WH Freeman Co, San Francisco; Merrifield J (1963) J. Am. Chem. Soc. 85:2149-2154; see also the Examples section herein). Exemplary automated systems include the Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer, Foster City, CA). In addition, there are many commercial providers of peptide synthesis services. If desired, subsequences can be chemically synthesized separately, and combined using chemical methods to provide full-length polypeptides.

PRODUCTION OF APTAMERS AND ANTIBODIES

[0119] Aptamers can be selected, designed, etc for binding various ligands (e.g., substrates in a first or second state) by methods known in the art. For example, aptamers are reviewed in Sun S "Technology evaluation: SELEX, Gilead Sciences Inc" Curr. Opin. Mol. Ther. 2000 Feb;2(1):100-5; Patel DJ, Suri AK "Structure, recognition and discrimination in RNA aptamer complexes with cofactors, amino acids, drugs and aminoglycoside antibiotics" J. Biotechnol. 2000 Mar, 74(1):39-60; Brody EN, Gold L "Aptamers as therapeutic and diagnostic agents" J. Biotechnol. 2000 Mar, 74(1):5-13; Hermann T, Patel DJ "Adaptive recognition by nucleic acid aptamers" Science 2000 Feb 4, 287(5454):820-5; Jayasena SD

"Aptamers: an emerging class of molecules that rival antibodies in diagnostics" Clin. Chem. 1999 Sep, 45(9):1628-50; and Famulok M, Mayer G "Aptamers as tools in molecular biology and immunology" Curr. Top. Microbiol. Immunol. 1999, 243:123-36.

[0120] Antibodies, e.g., that recognize the first or second state of a substrate, can likewise be generated by methods known in the art. For the production of antibodies to a particular polypeptide (e.g., for use as a detection module), various host animals may be immunized by injection with the polypeptide or a portion thereof. Such host animals include, but are not limited to, rabbits, mice and rats, to name but a few. Various adjuvants may be used to enhance the immunological response, depending on the host species; adjuvants include, but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum.*

[0121] Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as a protein or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals, such as those described above, may be immunized by injection with the protein, or a portion thereof, supplemented with adjuvants as also described above. The protein can optionally be produced and purified as described herein. For example, recombinant protein can be produced in a host cell, or a synthetic peptide derived from the sequence of the protein can be conjugated to a carrier protein and used as an immunogen Standard immunization protocols are described in, e.g., Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York. Additional references and discussion of antibodies is also found herein.

[0122] Monoclonal antibodies (mAbs), which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein (Nature 256:495-497, 1975; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al. (1983) Immunology Today 4:72; Cole et al (1983) Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al. (1985) Monoclonal Antibodies and Cancer Therapy, Alan R Liss, Inc., pp 77-96). Such antibodies may be of any immunoglobulin class, including IgG, IgM, IgE, IgA, IgD, and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*.

[0123] In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al. (1984) Proc. Natl. Acad. Sci. USA 81:6851-6855; Neuberger et al. (1984) Nature 312:604-608; Takeda et al (1985) Nature 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity, can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable or hypervariable region derived from a murine mAb and a human immunoglobulin constant region.

[0124] Similarly, techniques useful for the production of "humanized antibodies" can be adapted to produce antibodies to the proteins, fragments or derivatives thereof. Such techniques are disclosed in U.S. Patent Nos 5,932,448; 5,693,762; 5,693,761; 5,585,089; 5,530,101; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,661,016; and 5,770,429.

[0125] In addition, techniques described for the production of single-chain antibodies (U.S. Patent No 4,946,778; Bird (1988) Science 242:423-426; Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al. (1989) Nature 334:544-546) can be used. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single-chain polypeptide.

[0126] Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to, the $F(ab')_2$ fragments, which can be produced by pepsin digestion of the antibody molecule, and the Fab fragments, which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al. (1989) Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

[0127] A large number of antibodies are commercially available. For example, monoclonal and/or polyclonal antibodies against any of a large number of specific proteins (both modified, e.g., phosphorylated, and unmodified), against phosphoserine, against phosphothreonine, against phosphotyrosine, and against any phosphoprotein (ie, against phosphoserine, phosphothreonine and phosphotyrosine) are available, for example, from Zymed Laboratories, Inc (www (dot) zymed (dot) com), QIAGEN, Inc (www (dot) qiagen (dot) com) and BD Biosciences (www (dot) bd (dot) com), among many other sources. In addition, a number of companies offer services that produce antibodies against the desired antigen (e.g., a protein supplied by the customer or a peptide synthesized to order), including

Abgent (www (dot) abgent (dot) com), QIAGEN, Inc (www (dot) merlincustomservices (dot) com) and Zymed Laboratories, Inc.

EXAMPLES

[0128] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. Accordingly, the following examples are offered to illustrate, but not to limit, the claimed invention.

Example 1: Deep Quench: An Expanded Dynamic Range for Protein Kinase Sensors

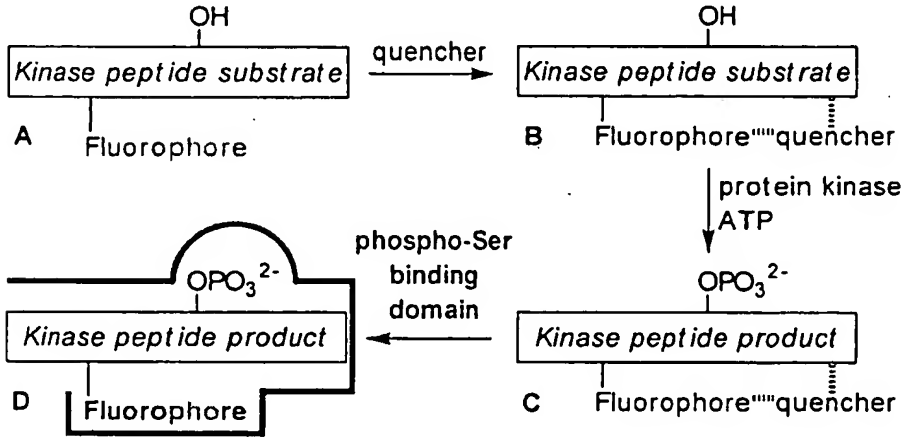
[0129] The following sets forth a series of experiments that demonstrate synthesis and use of exemplary sensors, including exemplary kinase sensors that include a fluorescent labeled substrate module, a quencher, and a detection module.

[0130] Protein kinases catalyze the phosphorylation of serine, threonine, and tyrosine residues in protein and peptide substrates. These enzymes have received considerable attention due to the relationship between aberrant kinase activity and an assortment of human afflictions. Specific and highly sensitive protein kinase sensors furnish, e.g., a means to rapidly identify inhibitors, assess protein structure/function relationships, and correlate kinase activity with cellular behavior. A large number of kinase assays have been described; however, assays with fluorescent readouts are most easily applied to both *in vitro* and intracellular settings GFP-labeled protein and fluorophore-labeled peptide substrates generally deliver, upon phosphorylation, a fluorescent response that varies from 10-60% to 2-9-fold, respectively[1]. By comparison, many fluorescent sensors developed for a variety of biomolecules (eg proteinases[2] and the detection of specific nucleotide sequences[3]) display enhancements of 25-fold and greater. A large dynamic range offers enhanced sensitivity, thereby furnishing a means to assess target biomolecule behavior under a variety of conditions. Unlike nearly all of the protein kinase assays reported to date,[4] the readout described in studies with proteinases[2] and molecular beacons[3] arise via relief of fluorescent quenching. We report herein an approach, devised around relief of fluorescent quenching, which delivers a robust protein kinase-elicited fluorescent response.

[0131] Initial studies focused on the strategy outlined in Scheme 1 A fluorophore-labeled serine kinase substrate (**A**) exhibits little or no fluorescence (**B**) in the presence of a quencher molecule. Upon phosphorylation, the peptide product (**C**) is sequestered by a phospho-Ser

binding domain to form the complex **D**, which disrupts the interaction between peptide-fluorophore and quencher. The latter partially or completely restores the fluorescence of the starting peptide.

Scheme 1. Enhanced sensing of protein kinase activity via a deeply quenched kinase peptide substrate.

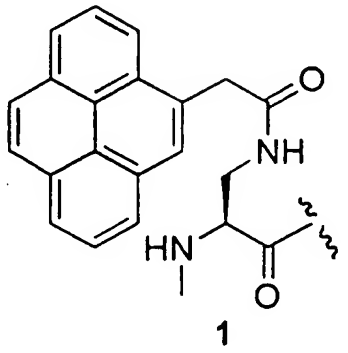


[0132] Pyrene was chosen to serve as the fluorophore on an amino acid sequence (AcGRTGRRFSYP-amide) (SEQ ID NO: 15) recognized by the cAMP-dependent protein kinase ("PKA")[5]. We employed the phospho-Ser binding domain, 14-3-3τ, to serve as the sequestering agent since 14-3-3 domains display a high affinity for phosphoSer-containing peptides (K_D < 100 nM)[6]. The assay was constructed in the following stages.

[0133] *Identification of quenching agents* Fluorescent quenching by a secondary dye is a commonly employed method used to study a wide assortment of biological phenomena[7]. However, without limitation to any particular mechanism, we screened for a molecule that would quench fluorophore fluorescence by forming a noncovalent complex with a targeted protein kinase peptide substrate. A library of 47 commercially available dyes (Supporting Information) was assembled and analyzed for the ability to quench the fluorescence of a family of pyrene-substituted peptides **P1** – **P11** (Table 1). Pyreneacetic acid (Pyr) was attached at different sites along the PKA consensus sequence peptide via a substituted 2,3-diaminopropionic ("Dap") residue **1** as well as to the N-terminus of the peptide via variable length linkers.

Table 1. Pyrene-substituted peptides **P1** – **P11** (SEQ ID NOs:1-11) containing either Dap (Pyr) at the indicated internal sites (**P1** – **P5**) (SEQ ID NO:1-5) or 1-pyreneacetyl appended to the N-terminus of peptides **P6** – **P11** (SEQ ID NO:6-11).

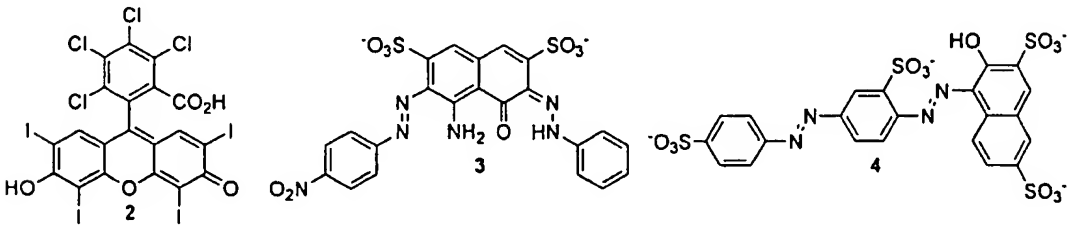
	Peptide
P1	Ac-GRTGRRFSDap(Pyr)P-amide
P2	Ac-GRTGRRDap(Pyr)SYP-amide
P3	Ac-GRTDap(Pyr)RRFSYP-amide
P4	Ac-GRDap(Pyr)GRRFSYP-amide
P5	Ac-Dap(Pyr)RTGRRFSYP-amide
P6	Pyr-ßAla-GRTGRRFSYP-amide
P7	Pyr-Abu-GRTGRRFSYP-amide
P8	Pyr-Ava-GRTGRRFSYP-amide
P9	Pyr-Ahx-GRTGRRFSYP-amide
P10	Pyr-Aoc-GRTGRRFSYP-amide
P11	Pyr-miniPEG™-GRTGRRFSYP-amide



1

[0134] Ten dyes were identified that serve as effective quenchers (≥40%) of pyrene fluorescence (5 μM peptide and 5 μM dye) for several of the peptides (Supporting Information), including Rose Bengal (**2**), Aniline Blue WS (**3**), and Ponceau S (**4**) (Chart 1). The latter, as well as the other lead quenchers, are negatively charged species. Complex formation of the quencher with the peptide is likely stabilized by electrostatic (positively charged Arg residues) and hydrophobic (fluorophore) interactions.

Chart 1. Lead quenching dyes of pyrene-peptide fluorescence.



[0135] K_D values were acquired for the set of the ten lead quenchers with peptide **P2**, in order to obtain a target range for the quencher:peptide ratios to be employed in the subsequent assays (*vide infra*). These apparent K_D values were determined using the

quenching of pyrene fluorescence as a barometer of peptide/quencher complex stability. Since the peptide/quencher pairs may interact via several different modes, not all of which might furnish efficient quenching, the actual K_Ds could be tighter than suggested by the apparent dissociation constants K_Ds with peptide **P2** range from 2.8 ± 0.8 μM (Evans Blue) up to 19.6 ± 3.4 μM (Reactive Blue) (Supporting Information). An inner filter effect (at high dye concentrations) was corrected as previously described[8].

[0136] *2 Identification of the lead pyrene-peptide/quencher pair.* The eleven pyrene-substituted peptides (**P1 – P11** at 5 μM) were incubated with a 5-, 10-, 25-, and 50-fold molar excess of each of the ten lead quenchers in the presence of PKA, ATP, and the phospho-Ser binding domain 14-3-3τ. Several control experiments were performed, including conducting the assay in the absence of quenching agent. Under the latter conditions, only small enhancements in fluorescence (0 – 64%) were observed (Supporting Information). Since pyrene is an environmentally sensitive fluorophore, these results suggest that the phosphopeptide product binds to 14-3-3 in a manner that inserts pyrene into a modestly hydrophobic environment. At high molar excess dye ratios (>25-fold), pyrene emission is so deeply quenched that background fluorescence significantly contributes to the total fluorescence of the pyrene-peptide sample. Consequently, the background was subtracted from all readings to establish a baseline upon which changes in fluorescence intensity could be quantified (Supporting Information).

[0137] Screening, using a multiwell plate reader, revealed several unique quencher/peptide pair combinations that exhibit robust fluorescence changes in response to phosphorylation: Aniline Blue WS **3** and **P9** peptide, Ponceau S **4** and **P2** peptide, and Rose Bengal **2** and **P5** peptide. A more detailed analysis was performed using a standard spectrofluorimeter (Fig. 1). The Rose Bengal/peptide **P5** pair exhibits an unprecedented 64-fold phosphorylation-induced enhancement in fluorescence. The Aniline Blue WS/peptide P9 combination is nearly as robust (55-fold), while the Ponceau S/peptide **P2** pair is somewhat more subdued (21-fold). The apparent K_Ds of the two most effective pairs (Rose Bengal/peptide **P5**: 0.40 ± 0.03 μM; Aniline Blue WS/peptide **P9**: 0.60 ± 0.03 μM) are significantly tighter than those obtained for the ten lead dyes with peptide **P2** (Supporting Information).

[0138] The peptides **P2** (K_m = 7.1 ± 1.9 μM; V_{max} = 8.4 ± 1.2 μmol/min-mg), **P5** (K_m = 1.7 ± 0.4 μM; V_{max} = 5.7 ± 0.4 μmol/min-mg), and **P9** (K_m = 1.6 ± 0.9 μM; V_{max} = 7.1 ± 1.2 μmol/min-mg) are all effective PKA substrates in the presence of 14-3-3τ. In addition, we

employed the Ponceau S/peptide **P2** combination to examine the inhibitory efficacy of the ATP analogue H9[9] and a peptide fragment (14-22) of PKI[10], a protein-based inhibitor of PKA. Under previously reported conditions ([ATP] = 10 μM), H9 is a reasonably effective inhibitor (IC_{50} = 1.9 ± 0.2 μM) of PKA. However, these conditions are nonphysiological since intracellular levels of ATP are typically above 1 mM. Under the latter conditions ([ATP] = 1 mM), the potency of H9 is dramatically reduced (IC_{50} = 42 ± 1 μM), as expected for an ATP analogue. In addition, we examined the inhibitory efficacy of the PKI 14-22 peptide inhibitor under identical conditions using two different assays. Both the Deep Quench strategy (1.1 ± 0.1 μM) and the commonly employed radioactive ATP method (1.6 ± 0.2 μM) furnish nearly identical IC_{50} values.

[0139] In summary, we have established a new approach for eliciting robust fluorescent readouts of protein kinase activity

[0140] ACKNOWLEDGMENTS We thank Dr Hsien-ming Lee for a gift of PKA and Dr Melanie Priestman for acquiring the IC_{50} value of the PKI peptide (radioactive method).

1. Rothman, D. M.; Shults, M. D.; Imperiali, B. *Trends Cell Biol.*, **2005**, *15*, 502-10.
2. For example, see Matayoshi, E. D.; Wang, G. T.; Krafft, G. A.; Erickson, J. *Science*, **1990**, *247*, 954-8.
3. Tan, W.; Wang, K.; Drake, T. J. *Cur. Opin. Chem. Biol.* **2004**, *8*, 547-53.
4. A few tyrosine kinase sensors that operate via an unquenching mechanism have been described: (a) Sun, H.; Low, K. E.; Woo, S.; Noble, R. L.; Graham, R. J.; Connaughton, S. S.; Gee, M. A.; Lee, L. G. *Anal. Chem.*, **2005**, *77*, 2043-9. (b) Wang, Q.; Cahill, S. M.; Blumenstein, M.; Lawrence, D. S. *J. Amer. Chem. Soc.*, **2006**, *128*, 1808-9. (c) Wang, Q.; Dai, Z.; Cahill, S. M.; Blumenstein, M.; Lawrence, D. S. *J. Amer. Chem. Soc.*, **2006**, *128*, 14016-7.
5. (a) Mitchell, R. D.; Glass, D. B.; Wong, C.-W.; Angelos, K. L., Walsh, D. A. *Biochemistry*, **1995**, *34*, 528-34. (b) A FRET-based PKA sensor has been described involving the intramolecular association of a phosphorylated amino acid sequence with 14-3-3 domain: Zhang, J.; Ma, Y.; Taylor, S. S.; Tsien, R. Y. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 14997-5002.
6. Yaffe, M. B.; Rittinger, K.; Volinia, S.; Caron, P. R.; Aitken, A.; Leffers, H.; Gamblin, S. J.; Smerdon, S. J.; Cantley, L. C. *Cell*, **1997**, *91*, 961-71.
7. M. K. Johansson *Methods Mol. Biol.* **2006**, *335*, 17-29.
8. Levine, R. L. *Clin. Chem.* **1977**, *23*, 2292-301.
9. Hidaka, H.; Inagaki, M.; Kawamoto, S.; Sasaki, Y. *Biochemistry*, **1984**, *23*, 5036-41.

10. Glass, D. B.; Cheng, H.-C.; Mende-Mueller, L.; Reed, J.; Walsh, D. A. *J. Biol. Chem.* **1989**, *264*, 8802-10.

SUPPORTING INFORMATION
EXPERIMENTAL SECTION

[0141] General reagents and solvents were purchased from Fisher or Aldrich. CLEAR Rink amide resin and Fmoc-2,6-dioxoaminooctanoic acid, HCTU [1H-benzotriazolium 1-[*bis*(dimethylamino)methylene]-5-chloro-,hexafluorophosphate (1-),3-oxide], and HOBt-Cl (6-chloro-1-hydroxy-1H-benzotriazole) were purchased from Peptides International (Louisville, KY). Fmoc-ßAla-OH, Fmoc-aminobutyric acid, Fmoc-aminovaleric acid, Fmoc-aminohexanoic acid, and Fmoc-aminooctanoic were purchased from Advanced Chem Tech (Louisville, KY). Fmoc-Dap(Mtt)-OH was purchased from Novabiochem (La Jolla, CA). PKA murine catalytic subunit plasmid and the GST-14-3-3τ plasmid (Aitken (2006) Semin. Cancer Biol. 16:162-72) were generous gifts from Dr. Susan Taylor and Dr. Alistair Aitken, respectively.

[0142] *Synthesis of Peptide Libraries* Peptides were synthesized by standard solid phase synthesis using Fmoc chemistry. The Fmoc protecting group was removed with 20% piperidine in dimethylformamide (DMF) (1x5 min, 1x20 min). Sequential coupling of Fmoc protected amino acids was achieved with 3 equiv Fmoc amino acid, 3 equiv HCTU, 3 equiv HOBt-Cl, and 6 equiv diisopropylethylamine (DIPEA). Completion of each reaction was monitored with the Kaiser and chloranil tests. Resins were washed between steps with DMF, isopropyl alcohol (IPA), and DCM. For peptides P1 - P5, the free N-terminal Gly^1 was acylated with 20 equiv of acetic anhydride in dissolved in 1:1 pyridine:DMF. The 4-methyltrityl protecting group on Dap(Mtt) was orthogonally removed using 5% trifluoroacetic acid (TFA) and 5% triisopropylsilane (TIPS) in DCM (5 min incubation). The resulting free ß-amine was acylated with 3 equiv 1-pyreneacetic acid in DMF containing 3 equiv HCTU, 3 equiv HOBt-Cl, and 6 equiv of DIPEA. The free N-termini of peptides P6 - P11 were directly acylated with 1-pyreneacetic acid following the Fmoc deprotection of terminal ß-alanine (ßAla), aminobutyric acid (Abu), aminovaleric acid (Ava), aminohexanoic acid (Ahx), aminooctanoic acid (Aoc), and amino-3,6-dioxoaminooctanoic acid (miniPEG^{TM}) groups, respectively. The remaining orthogonal protecting groups were removed and the peptides cleaved from their resins with 95% TFA, 5% water, 5% TIPS (3 hr). The peptides were isolated via filtration of the resin, precipitation with ice-cold diethyl ether, and centrifugation. The precipitates were air dried and purified by reverse-phase HPLC using a linear gradient (3% - 40% acetonitrile in water with 0.1% TFA over 40 min). The peak

corresponding to the desired peptide was collected, frozen, and lyophilized. The resulting white, flocculent peptides were characterized by electrospray ionization mass spectrometry: **P1** Ac-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Dap(Pyr)-Pro-amide (SEQ ID NO:1) (m/z calculated 1403.72, found 1403.80); **P2** Ac-Gly-Arg-Thr-Gly-Arg-Arg-Dap(Pyr)-Ser-Tyr-Pro-amide (SEQ ID NO:2) (m/z calculated 1419.72, found 1419.60); **P3** Ac-Gly-Arg-Thr-Dap(Pyr)-Arg-Arg-Phe-Ser-Tyr-Pro-amide (SEQ ID NO:3) (m/z calculated 1507.75, found 1509.47); **P4** Ac-Gly-Arg-Dap(Pyr)-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (SEQ ID NO:4) (m/z calculated 1463.72, found 1464.87); **P5** Ac-Dap(Pyr)-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (SEQ ID NO:5) (m/z calculated 1507.75, found 1509.93); **P6** Pyr-ßAla-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (SEQ ID NO:6) (m/z calculated 1507.75, found 1509.47); **P7** Pyr-Abu-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (SEQ ID NO:7) (m/z calculated 1521.76, found 1523.80); **P8** Pyr-Ava-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (SEQ ID NO:8) (m/z calculated 1535.78, found 1537.40); **P9**, Pyr-Ahx-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (SEQ ID NO:9) (m/z calculated 1549.79, found 1551.60); **P10** Pyr-Aoc-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (SEQ ID NO:10) (m/z calculated 1577.83, found 1578.73); **P11** Pyr-miniPEG™-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (SEQ ID NO:11) (m/z calculated 158276, found 158373).

[0143] *Identification of Lead Quencher Dyes* The concentration of peptides **P1 – P11** was adjusted to 50 μM based on the molar excitation coefficient of 22,000 M^{-1} cm^{-1} at 345 nm. The concentrations of 47 dyes were adjusted to 50 μM by weight. The peptides were screened against the dyes on 96 well plates using an HTS 7000 Bio Assay Reader (Perkin Elmer) with 340 nm excitation filter and 380 nm emission filter, a setting of 100 μs integration time, and 5 flashes. Each well contained 5 μM peptide and 5 μM dye in 50 mM Tris-HCl at pH 75 Dyes that resulted in the greatest degree of fluorescence quenching were noted.

Chart S1. Lead quencher dyes of pyrene peptides **P1 – P11.**

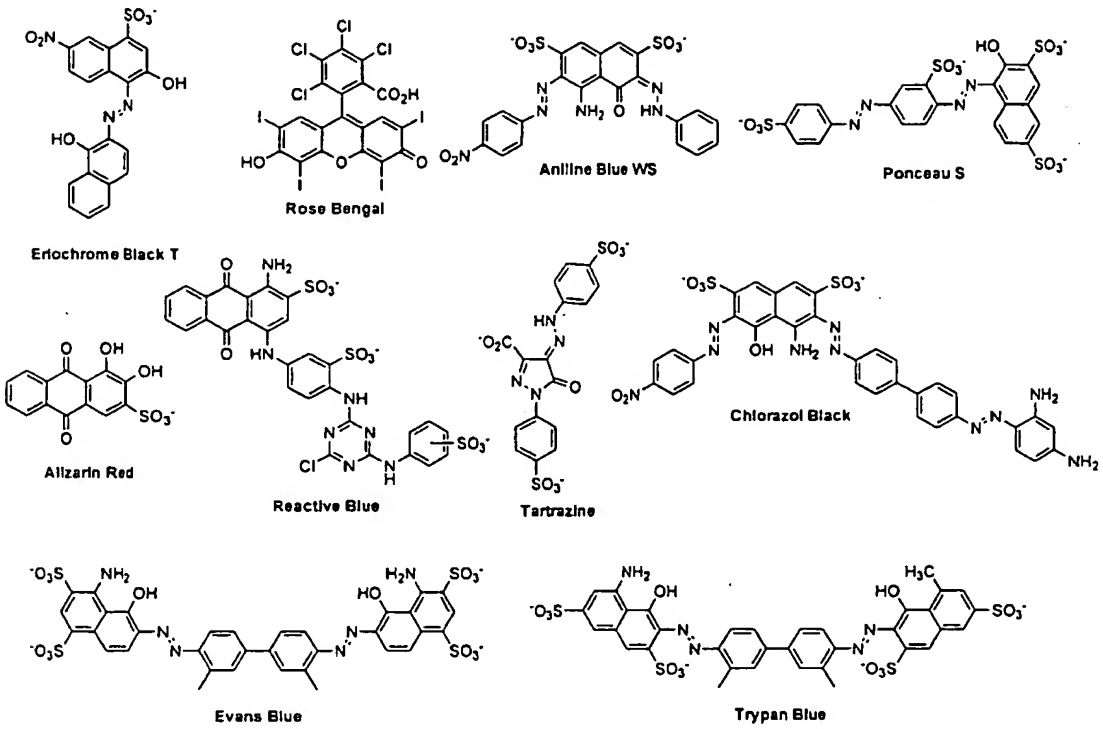


Table S1. Library of Dyes.

D1	Acid Green 27
D2	Acid Blue 40
D3	Evans Blue
D4	Acid Alizarin Violet N
D5	Acid Blue 80
D6	Reactive Blue 2
D7	N,N-dimethylnitrosoaniline
D8	Cresol Red
D9	Phenol Red
D10	Methyl Orange
D11	Bromophenol Blue
D12	BUFFER
D13	Xylene Cyanol FF
D14	Disperse Yellow 3
D15	Ethyl Orange
D16	Methylene Blue
D17	Brilliant Blue R
D18	Eriochrome Black T
D19	Alizarin Red
D20	Malachite Green oxalate
D21	Phenolphthalein
D22	Carminic Acid

D23	Nuclear Fast Red
D24	Acid Fuchsin
D25	Acridine Orange
D26	Acridine Yellow G
D27	Aniline Blue WS
D28	Azure A
D29	Azure B bromide
D30	Basic Fuchsin
D31	Bismark Brown Y
D32	Brilliant Yellow
D33	Bromocresol Purple
D34	Chlorazol Black E
D35	Chlorophenol Red
D36	Chrysoidine Y
D37	Erythrosin
D38	Ethyl Violet
D39	Naphthol Blue Black
D40	Methylthymol Blue
D41	Methyl Violet
D42	Ponceau S
D43	Rose Bengal
D44	Rosolic Acid
D45	Safranin O
D46	Serva Violet 49
D47	Tartrazine
D48	Trypan Blue

[0144] *Acquisition of Apparent K_D Values for Lead Quencher Dyes with Peptide P2* (Figure 2). Varying concentrations of 10 dyes, ranging from 0.5 – 500 μM, were added to 5 μM pyrene-labeled **P2** peptide in 100 mM Tris HCl pH 75 buffer (96 well plates). A Spectra Max Gemini EM plate reader (Molecular Devices) was used for fluorescence measurements (λ_{ex} = 342 nm and (λ_{em} = 380 nm). Correction for the inner filter effect was made using the antilogarithm of the effective optical density times half the width of the fluorescence well as previously reported (Clin. Chem. 23 (12) 2292-2301, 1977). Molar absorbtivities (ε_{342} and ε_{380}) were calculated from single absorbance spectra at a [dye] = 7.81 μM. For all dyes at concentrations below 10 μM, the inner filter effect required a correction of less than 10% in the measured fluorescence. However, at higher concentrations, the effect became significant for strongly absorbing dyes. After correcting for the inner filter effect, the percentage of quench was plotted against the concentration of the dye. A nonlinear regression analysis fit of the data to the rectangular hyperbola model using the Sigma Plot version 8.02 software was used to obtain apparent K_D values.

Table S2. Apparent K_D Values of Lead Quenchers with Peptide P2.

QUENCHER DYE		Apparent K_D (μM)
D3	Evans Blue	2.8 ± 0.8
D6	Reactive Blue	19.6 ± 3.4
D18	Eriochrome Black	14.3 ± 3.3
D19	Alizarin Red	7.3 ± 2.5
D27	Aniline Blue WS	18.1 ± 2.6
D34	Chlorazol Black E	7.7 ± 1.5
D42	Ponceau S	11.2 ± 2.7
D43	Rose Bengal	7.5 ± 1.6
D47	Tartrazine	15.0 ± 2.1
D48	Trypan Blue	11.9 ± 3.6

[0145] Acquisition of apparent K_D values for lead quencher/peptide pairs were performed as described above. In addition, the apparent K_D value for the *phosphorylated* **P5** peptide AcDap(Pyr)RTGRRFS(PO_3^{2-})YP-amide with Rose Bengal is 210 ± 40 nM, slightly tighter than that found for the unphosphorylated AcDap(Pyr)RTGRRFSYP-amide/Rose Bengal pair (400 ± 30 nM) (Figure 3).

[0146] *Screening of Lead Quenching Dyes 1 – 10 with Peptides P1 - P1.* PKA-catalyzed phosphorylation was initiated by addition of 25 μL of 100 nM PKA enzyme to the following solution: 25 μL 50 μM fluorescent peptide substrates (**P1 – P11**), 25 μL 20 mM DTT, 25 μL 10 mM ATP, 25 μL 50 mM $MgCl_2$, 25 μL 100 μM 14-3-3τ, 25μL 0.5 M Tris HCl pH 7.5, 25 μL dye (10 dyes at 4 concentrations, 0.25 mM, 0.5 mM, 1.25 mM 2.5 mM and no dye as a control) to give final volume of 250 μL. The concentrations per well were: 10 nM PKA, 5 μM peptide, 10 μM 14-3-3τ, 1 mM ATP, 5 mM $MgCl_2$, 2 mM DTT, and 25 μM, 50 μM, 250 μM or 500 μM each of 10 different lead dyes in 50 mM Tris at pH 7.5 buffer. The HTS 7000 Bio Assay Reader was set in kinetic mode to monitor the progress of reaction (340 nm excitation filter, 380 nm emission filter, 100 μs and 5 flashes).

Table S3. Control Experiment: Phosphorylation-induced Change in Fluorescence of Pyrene-labeled peptides **P1** - **P11** (SEQ ID NOs:1-11) in the Absence of Quenching Dye.

PYRENE-LABELED PEPTIDE		% Fluorescence Enhancement
P1	Ac-GRTGRRFSDap(Pyr)P-amide	0
P2	Ac-GRTGRRDap(Pyr)SYP-amide	51%
P3	Ac-GRTDap(Pyr)RRFSYP-amide	19%
P4	Ac-GRDap(Pyr)GRRFSYP-amide	40%
P5	Ac-Dap(Pyr)RTGRRFSYP-amide	64%
P6	Pyr-ßAla-GRTGRRFSYP-amide	49%
P7	Pyr-Abu-GRTGRRFSYP-amide	47%
P8	Pyr-Ava-GRTGRRFSYP-amide	31%
P9	Pyr-Ahx-GRTGRRFSYP-amide	48%
P10	Pyr-Aoc-GRTGRRFSYP-amide	39%
P11	Pyr-miniPEG™-GRTGRRFSYP-amide	38%

[0147] *Beer's Law Analysis* The fluorescence intensities of different concentrations of phosphorylated **P5** peptide (ranging from 0 to 1 μM and incubated with 10 μM 14-3-3τ and 100 mM Tris HCl pH 7.5) were determined in the presence of 12.5 μM Rose Bengal. The intensities were plotted against the peptide concentration and the data fit to a straight line. The fit of the data with background correction is shown as a dotted line (Figure 4). The background was acquired by using a sample that had all the assay components except the fluorophore-peptide by using the "Acquire Background" mode in FeliX software (Photon Technology version 1.42). This background intensity was automatically subtracted from subsequent measurements by the software.

[0148] *Acquisition of K_m and V_{max} values*: Phosphorylation dependent increase in pyrene fluorescence intensity of peptides **P2**, **P5** and **P9** were monitored on a Photon Technology QM-1 spectrofluorimeter at 30 °C using 343 nm excitation wavelength, 380 nm emission wavelength, and an 8 nm slit-width. After equilibration of different concentrations of the pyrene-labeled peptide substrate with 50 mM Tris buffer pH 7.5, 30 μM 14-3-3τ, 1 mM ATP, 5 mM $MgCl_2$, 2 mM DTT, for 10 min, 10 nM enzyme was added and the reaction progress curves obtained. Reaction rates were determined from the slope under conditions where 5 - 8% substrate had been converted to product in duplicate. The resulting slopes (initial velocity, v_o) for each of the progress curves were plotted versus the concentration of

substrate. A nonlinear regression analysis was used to fit the data to the rectangular hyperbola model using the Sigma Plot version 8.02 software.

[0149] *Assay Dependence on 14-3-3τ:* Phosphorylation-dependent increase in pyrene fluorescence intensity of peptide **P5**, in the presence and absence of 14-3-3τ, was monitored on a Photon Technology QM-1 spectrofluorimeter at 30 °C using 343 nm excitation wavelength, 380 nm emission wavelength with an 8 nm slit-width. 5 μM pyrene-labeled peptide substrate **P5** was pre-incubated in 25 μM Rose Bengal, 5 mM $MgCl_2$, 2 mM DTT, 1.4 μM PKA, and 50 mM Tris buffer pH 7.5, in the presence, and absence, of 30 μM 14-3-3τ, at 30 °C for 5 min (Figure 5). After 1 min, 1 mM ATP was added and the reaction progress followed. In the absence of 14-3-3τ (Figure 5, lower trace), no change in fluorescence intensity was observed.

[0150] *Inhibitor IC_5 values* (Figure 6). 1 μM pyrene-labeled peptide substrate **P2** was incubated in 60 μM Ponceau S, 50 mM Tris buffer pH 7.5, 1 mM ATP, 30 μM 14-3-3τ, 5 mM $MgCl_2$, 2 mM DTT at 30 °C for 5 min. 10 nM PKA enzyme was added and the reaction progress followed for 1 min. This step was used to adjust for inter-assay variability and to verify that no significant drop in enzyme activity occurs over the course of the determinations. Subsequently, inhibitor was added at different concentrations. Reaction rates were measured under conditions where less than 10% substrate had been converted to product. Fractional velocities (v/v_0) were plotted against inhibitor concentration [I] and fit using the Sigma Plot version 8.02 software's four-parameter logistic nonlinear regression analysis. PKI (14-22) exhibits an IC_{50} value of 1.1 ± 0.1 μM. The IC_{50} value of PKI (14-22) using the standard radioactive ATP method is 1.6 ± 0.2 μM. H9•HCl exhibits an IC_{50} value of 42 ± 1 μM at 1 mM ATP and a value of 1.9 ± 0.2 μM at 10 μM ATP.

[0151] *Fluorescence change dependency on instrumentation/reading mode.* We have found that the phosphorylation-induced fluorescence change is dependent upon instrumentation and reading mode. In brief, the least dramatic changes are observed in a plate reader (Molecular Devices Spectra Max Gemini EM) using the bottom read mode (i.e., from below through the bottom of the clear well plates: Costar 3631 flat bottom 96 multiwell plates) A more robust change is obtained via a top read mode (Molecular Devices Spectra Max Gemini) for a multiwell plate (Wallac B & W isoplate 1450-582). The highest fluorescence fold change is provided using a dedicated spectrofluorimeter (Photon Technology QM-1) and a quartz cuvette as the sample holder. These results are summarized in Table S4 for the three lead peptide/quencher pairs. Initial screening of the library of

peptides **P1** – **P11** with the ten lead quenchers (Chart S1) was performed using the bottom read mode. A summary of these results is furnished in Figure 7.

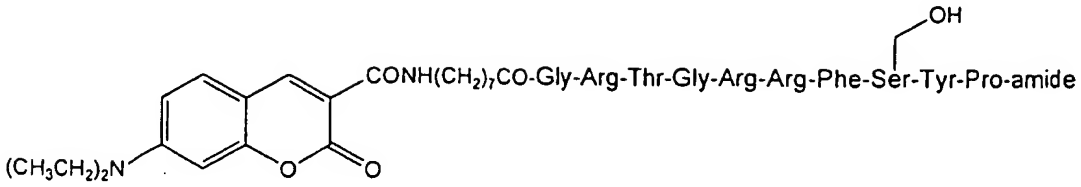
[0152] An example of assay conditions in plate reader mode is furnished for the top read with peptide **P5** and Rose Bengal: Phosphorylation-dependent increase in pyrene fluorescence intensity of peptide dye pair **P5**/Rose Bengal, was monitored on a Molecular Devices Spectra Max Gemini plate reader at 30 °C using 330 nm excitation wavelength, 380 nm emission wavelength. Three wells containing 5 μM pyrene-labeled peptide substrate **P5** were pre-incubated in 5 mM $MgCl_2$, 2 mM DTT, 1 mM ATP, and 50 mM Tris buffer pH 7.5, 30 μM 14-3-3τ and 25 μM Rose Bengal, at 30 °C for 10 min. 0.7 μM PKA was added and the reaction progress followed. Three additional wells containing all the assay components except for **P5** peptide were used for blank readings.

Table S4. Phosphorylation-induced fluorescence fold-change of lead peptide/quencher pairs as a function of instrumentation/read mode.

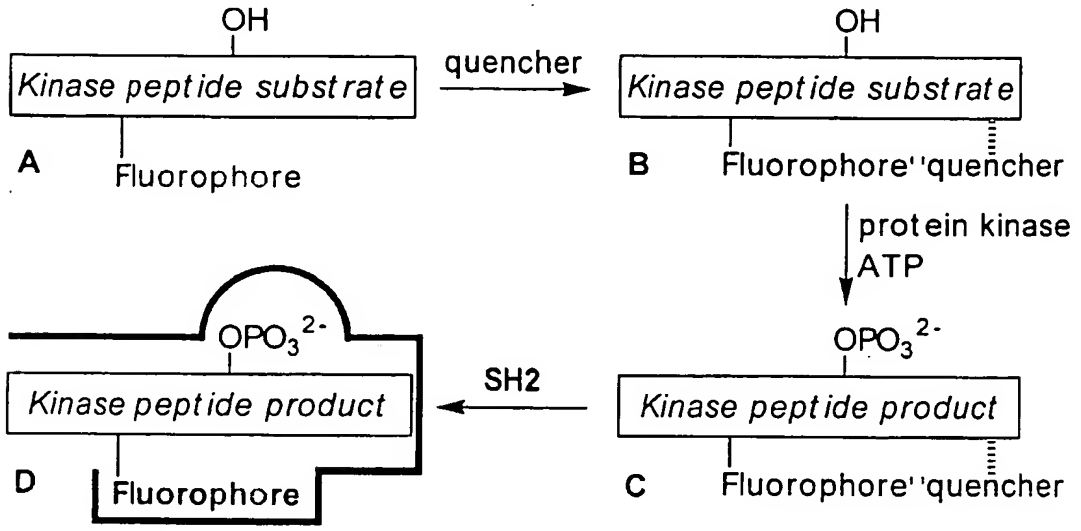
Peptide/Quencher (ratio)	Conditions	Fluorescence Change
P2/ Ponceau S (1:50)	Plate reader – bottom read	7-fold
	Plate reader – top read	15-fold
	spectrofluorimeter	21-fold
P5/Rose Bengal (1:5)	Plate reader – bottom read	8-fold
	Plate reader – top read	30-fold
	spectrofluorimeter	64-fold
P9/ Aniline Blue WS (1:10)	Plate reader – bottom read	9-fold
	Plate reader – top read	19-fold
	spectrofluorimeter	55-fold

[0153] *Additional exemplary sensors.* The coumarin-peptide **P12** shown below ($\lambda_{excitation}$ = 425 nm and $\lambda_{emission}$ = 470 nm), containing the coumarin derivative 7-diethylaminocoumarin-3-carboxylic acid, was also prepared. Using **P12** as the substrate module with an Acid Green 27 quencher and a 14-3-3 detection module, we observe a 225-fold enhancement in fluorescence (Figure 8). Concentrations in the assay were 16 μM peptide **P12**, 10 μM 14-3-3, and 30 μM Acid green 27.

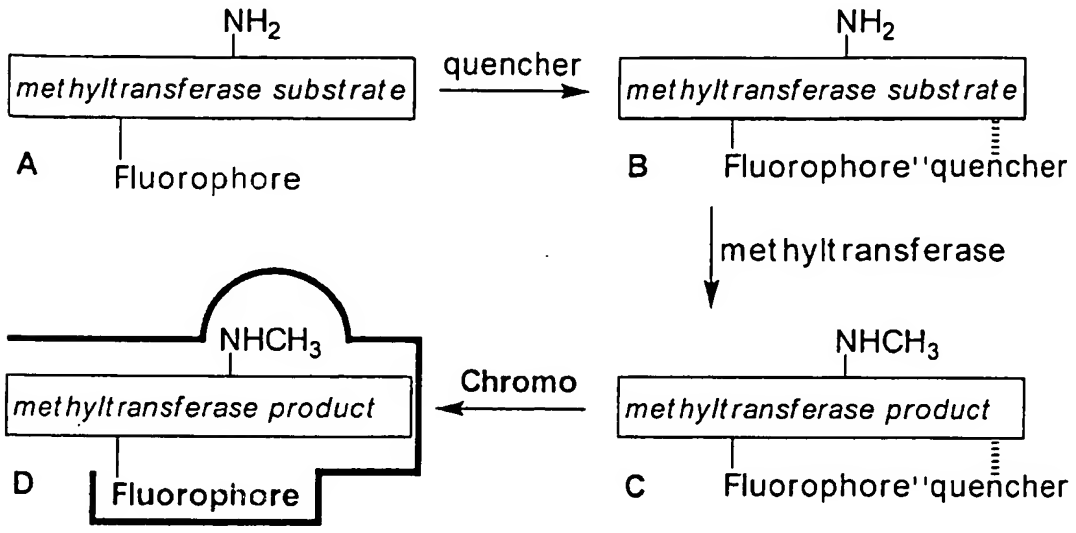
P12 (SEQ ID NO:12)



[0154] While the preceding examples have focused on serine kinase sensors, as noted above, the strategy outlined herein is applicable to other enzymes as well, for example: 1) tyrosine protein kinases, using SH2 or PTB domains to capture the phosphorylated product;

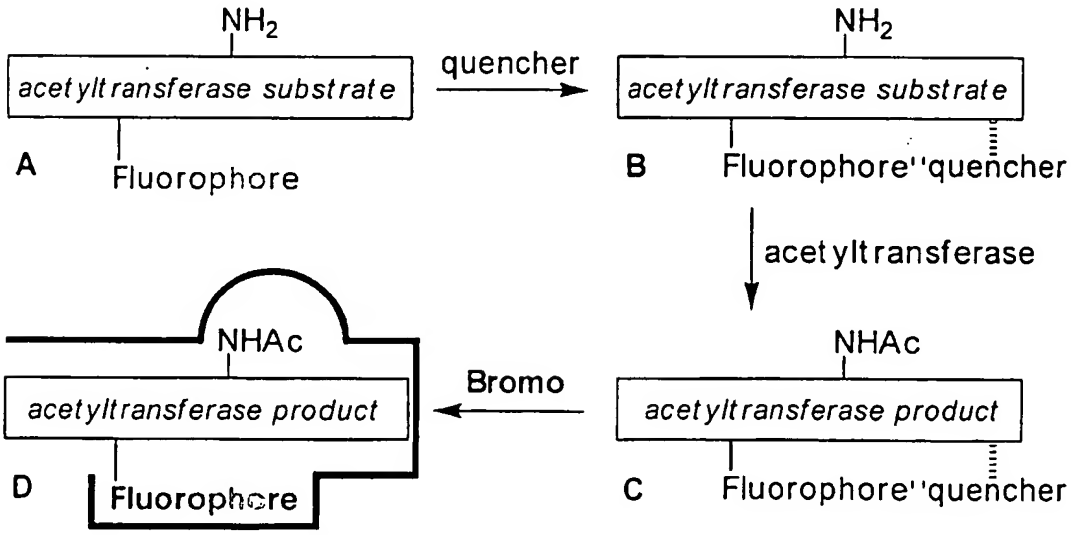


2) methyltransferases, eg, histone transferases with respect to epigenomics, using a Chromo domain to capture the methylated lysine peptide;

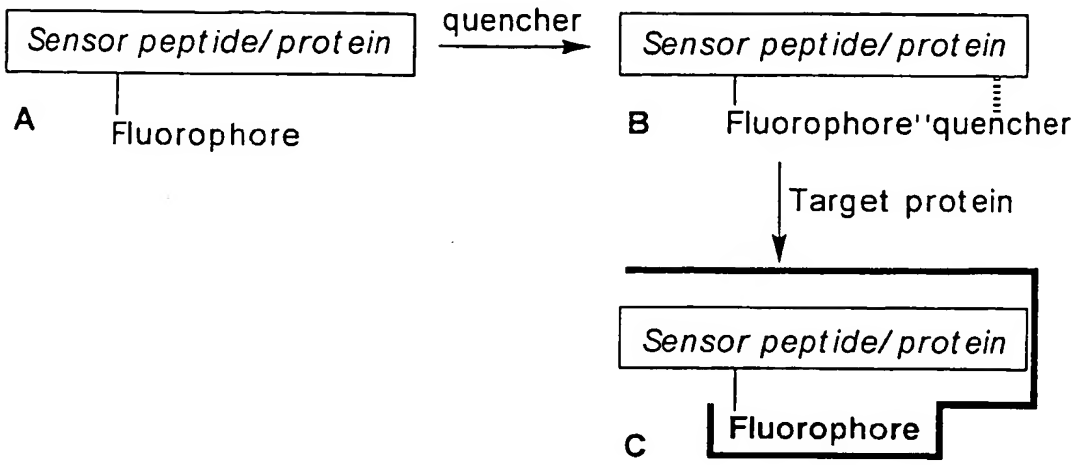


and

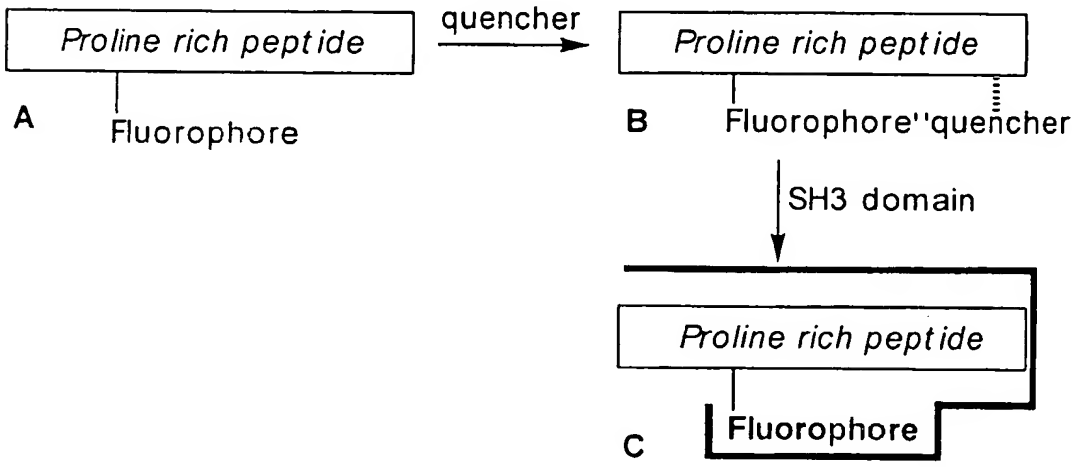
3) acetyltransferases, eg, histone acetyltransferases with respect to epigenomics, using a Bromo domain to capture the acetylated lysine peptide.



Also as noted, a general strategy is presented for any peptide or protein biosensor that binds to a target protein.



For example, interaction between a fluorophore-labeled proline rich peptide and an SH3 domain can be detected as schematically illustrated below.



[0155] While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques and apparatus described above can be used in various combinations. All publications, patents, patent applications, and/or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually indicated to be incorporated by reference for all purposes.

What is claimed is:

1 A composition comprising:

a sensor for detecting an activity of an enzyme, the sensor comprising

a) a substrate module comprising

i) a substrate for the enzyme, wherein the substrate is in a first state on which the enzyme can act, thereby converting the substrate to a second state, and

ii) a fluorescent label;

b) a detection module, which detection module binds to the substrate module when the substrate is in the first state, or which detection module binds to the substrate module when the substrate is in the second state; and

c) a quencher, wherein the quencher is not covalently bound to the substrate module or to the detection module;

wherein binding of the detection module to the substrate module results in an increased intensity of fluorescent emission from the label.

2 The composition of claim 1, comprising the enzyme.

3 The composition of claim 1, wherein the detection module binds to the substrate module when the substrate is in the second state.

4 The composition of claim 1, wherein the substrate module comprises a first molecule and the detection module comprises a second molecule.

5 The composition of claim 4, wherein the substrate module comprises a first polypeptide and the detection module comprises a second polypeptide.

6 The composition of claim 1, wherein the substrate is a polypeptide substrate.

7 The composition of claim 1, wherein the enzyme is a protein kinase, wherein the substrate in the first state is unphosphorylated, and wherein the substrate in the second state is phosphorylated.

8 The composition of claim 7, wherein the detection module binds to the substrate module when the substrate is in the second state.

9 The composition of claim 7, wherein the protein kinase is a serine/threonine protein kinase.

10 The composition of claim 9, wherein the substrate module comprises a first polypeptide and the detection module comprises a second polypeptide, the second polypeptide comprising a 14-3-3 domain or an antibody.

11 The composition of claim 7, wherein the protein kinase is a tyrosine protein kinase.

12 The composition of claim 11, wherein the substrate module comprises a first polypeptide and the detection module comprises a second polypeptide, the second polypeptide comprising an SH2 domain, a PTB domain, or an antibody.

13 The composition of claim 1, wherein the enzyme is a protein phosphatase, wherein the substrate in the first state is phosphorylated, and wherein the substrate in the second state is unphosphorylated.

14 The composition of claim 13, wherein the detection module binds to the substrate module when the substrate is in the first state.

15 The composition of claim 1, wherein the enzyme is a histone methyltransferase, a histone lysine methyltransferase, a histone arginine methyltransferase, or a protein lysine methyltransferase.

16 The composition of claim 15, wherein the substrate module comprises a first polypeptide and the detection module comprises a second polypeptide, the second polypeptide comprising a chromodomain or an antibody.

17 The composition of claim 1, wherein the enzyme is a histone acetyltransferase or a lysine acetyltransferase.

18 The composition of claim 17, wherein the substrate module comprises a first polypeptide and the detection module comprises a second polypeptide, the second polypeptide comprising a bromodomain or an antibody.

19 The composition of claim 1, wherein the quencher forms a non-covalent complex with the substrate module, which complex is disrupted upon binding of the detection module to the substrate module, thereby resulting in the increased intensity of fluorescent emission from the label.

20 The composition of claim 1, wherein the quencher forms a non-covalent complex with the substrate module with an apparent K_d of about 20 μM or less, about 10 μM or less, or about 1 μM or less.

21 The composition of claim 1, wherein the substrate module comprises a polypeptide substrate comprising amino acid sequence $X^{-4}\,R^{-3}\,R^{-2}\,X^{-1}\,S^{0}\,X^{+1}\,X^{+2}$ (SEQ ID NO:13);

where X^{-4} and X^{+2} are independently selected from the group consisting of: an amino acid residue and an amino acid residue comprising the fluorescent label; and

where X^{-1} and X^{+1} are independently selected from the group consisting of: a hydrophobic amino acid residue and an amino acid residue comprising the fluorescent label.

22 The composition of claim 1, wherein the substrate module is any one of **P1-P12** (SEQ ID NOs:1-12).

23 The composition of claim 1, wherein the detection module is a 14-3-3 domain, and wherein the substrate module is **P5** and the quencher is Rose Bengal, the substrate module is **P9** and the quencher is Aniline Blue WS, the substrate module is **P2** and the quencher is Ponceau S, or the substrate module is **P12** and the quencher is Acid Green 27.

24 The composition of claim 1, wherein the increased intensity of fluorescent emission from the label is an increase of at least about 7 fold, at least about 20 fold, at least about 50 fold, at least about 100 fold, or at least about 200 fold.

25 The composition of claim 1, wherein the label is pyrene or a coumarin derivative.

26 The composition of claim 1, wherein the quencher is selected from the group consisting of: Evans Blue, Reactive Blue, Eriochrome Black T, Alizarin Red, Aniline Blue WS, Chlorazol Black, Ponceau S, Rose Bengal, Tartrazine, Trypan Blue, and Acid Green 27.

27 The composition of claim 1, wherein when the substrate module is not bound to the detection module, the quencher quenches fluorescent emission by the label by at least about 40%, as compared to fluorescent emission in the absence of the quencher.

28 The composition of claim 1, wherein the molar ratio of the quencher to the substrate module in the composition is at least about 5 to 1, at least about 10 to 1, at least about 25 to 1, or at least about 50 to 1.

29 The composition of claim 1, wherein the sensor comprises one or more photolabile caging groups covalently bound to the substrate, which caging groups inhibit or prevent the enzyme from acting upon the substrate.

30 The composition of claim 1, comprising a modulator or potential modulator of the activity of the enzyme.

31 A method of assaying an activity of an enzyme, the method comprising:

contacting the enzyme with a sensor, the sensor comprising

a) a substrate module comprising

i) a substrate for the enzyme, wherein the substrate is in a first state on which the enzyme can act, thereby converting the substrate to a second state, and

ii) a fluorescent label;

b) a detection module, which detection module binds to the substrate module when the substrate is in the first state, or which detection module binds to the substrate module when the substrate is in the second state; and

c) a quencher, wherein the quencher is not covalently bound to the substrate module or to the detection module;

wherein binding of the detection module to the substrate module results in an increased intensity of fluorescent emission from the label;

detecting the increased intensity of fluorescent emission from the label; and

correlating the increased intensity of fluorescent emission from the label to the activity of the enzyme, thereby assaying the activity of the enzyme.

32 The method of claim 31, wherein the detection module binds to the substrate module when the substrate is in the second state.

33 The method of claim 31, wherein the substrate module comprises a first molecule and the detection module comprises a second molecule.

34 The method of claim 33, wherein the substrate module comprises a first polypeptide and the detection module comprises a second polypeptide.

35 The method of claim 31, wherein the substrate is a polypeptide substrate.

36 The method of claim 31, wherein the enzyme is a protein kinase, wherein the substrate in the first state is unphosphorylated, and wherein the substrate in the second state is phosphorylated.

37 The method of claim 36, wherein the detection module binds to the substrate module when the substrate is in the second state.

38 The method of claim 36, wherein the protein kinase is a serine/threonine protein kinase.

39 The method of claim 38, wherein the substrate module comprises a first polypeptide and the detection module comprises a second polypeptide, the second polypeptide comprising a 14-3-3 domain or an antibody.

40 The method of claim 36, wherein the protein kinase is a tyrosine protein kinase.

41 The method of claim 40, wherein the substrate module comprises a first polypeptide and the detection module comprises a second polypeptide, the second polypeptide comprising an SH2 domain, a PTB domain, or an antibody.

42 The method of claim 31, wherein the enzyme is a protein phosphatase, wherein the substrate in the first state is phosphorylated, and wherein the substrate in the second state is unphosphorylated.

43 The method of claim 42, wherein the detection module binds to the substrate module when the substrate is in the first state.

44 The method of claim 31, wherein the enzyme is a histone methyltransferase, a histone lysine methyltransferase, a histone arginine methyltransferase, or a protein lysine methyltransferase.

45 The method of claim 44, wherein the substrate module comprises a first polypeptide and the detection module comprises a second polypeptide, the second polypeptide comprising a chromodomain or an antibody.

46 The method of claim 31, wherein the enzyme is a histone acetyltransferase or a lysine acetyltransferase.

47 The method of claim 46, wherein the substrate module comprises a first polypeptide and the detection module comprises a second polypeptide, the second polypeptide comprising a bromodomain or an antibody.

48 The method of claim 31, wherein the quencher forms a non-covalent complex with the substrate module, which complex is disrupted upon binding of the detection module to the substrate module, thereby resulting in the increased intensity of fluorescent emission from the label.

49 The method of claim 31, wherein the quencher forms a non-covalent complex with the substrate module with an apparent K_d of about 20 μM or less, about 10 μM or less, or about 1 μM or less.

50 The method of claim 31, wherein the substrate module comprises a polypeptide substrate comprising amino acid sequence $X^{-4}R^{-3}R^{-2}X^{-1}S^{0}X^{+1}X^{+2}$ (SEQ ID NO:13);

where X^{-4} and X^{+2} are independently selected from the group consisting of: an amino acid residue and an amino acid residue comprising the fluorescent label; and

where X^{-1} and X^{+1} are independently selected from the group consisting of: a hydrophobic amino acid residue and an amino acid residue comprising the fluorescent label.

51 The method of claim 31, wherein the substrate module is any one of **P1-P12** (SEQ ID NOs:1-12).

52 The method of claim 31, wherein the detection module is a 14-3-3 domain, and wherein the substrate module is **P5** and the quencher is Rose Bengal, the substrate module is **P9** and the quencher is Aniline Blue WS, the substrate module is **P2** and the quencher is Ponceau S, or the substrate module is **P12** and the quencher is Acid Green 27.

53 The method of claim 31, wherein the increased intensity of fluorescent emission from the label is an increase of at least about 7 fold, at least about 20 fold, at least about 50 fold, at least about 100 fold, or at least about 200 fold.

54 The method of claim 31, wherein the label is pyrene or a coumarin derivative.

55 The method of claim 31, wherein the quencher is selected from the group consisting of: Evans Blue, Reactive Blue, Eriochrome Black T, Alizarin Red, Aniline Blue WS, Chlorazol Black, Ponceau S, Rose Bengal, Tartrazine, Trypan Blue, and Acid Green 27.

56 The method of claim 31, wherein when the substrate module is not bound to the detection module, the quencher quenches fluorescent emission by the label by at least about 40%, as compared to fluorescent emission in the absence of the quencher.

57 The method of claim 31, wherein the sensor comprises one or more caging groups associated with the substrate, which caging groups inhibit the enzyme from acting upon the substrate, the method comprising uncaging the substrate, thereby freeing the substrate from inhibition by the one or more caging groups.

58 The method of claim 57, wherein uncaging the substrate comprises exposing the substrate to light of a first wavelength.

59 The method of claim 31, comprising contacting the enzyme with a test compound, assaying the activity of the enzyme in the presence of the test compound, and comparing the activity of the enzyme in the presence of the test compound with the activity of the enzyme in the absence of the test compound.

60 A composition comprising:

a labeled polypeptide comprising a first polypeptide and a fluorescent label;

a second polypeptide to which the first polypeptide binds; and

a quencher, wherein the quencher is not covalently bound to the first polypeptide or to the second polypeptide;

wherein binding of the first polypeptide to the second polypeptide results in an increased intensity of fluorescent emission from the label.

61 The composition of claim 60, wherein the first polypeptide is a proline rich polypeptide and the second polypeptide comprises an SH3 domain, wherein the first polypeptide comprises a phosphorylated serine residue and the second polypeptide comprises a 14-3-3 domain, wherein the first polypeptide comprises a phosphorylated tyrosine residue and the second polypeptide comprises an SH2 or PTB domain, wherein the first polypeptide comprises a methylated lysine residue and the second polypeptide comprises a chromodomain, or wherein the first polypeptide comprises an acetylated lysine residue and the second polypeptide comprises a bromodomain.

62 The composition of claim 60, wherein the first polypeptide comprises amino acid sequence $X^{-4}\,R^{-3}\,R^{-2}\,X^{-1}\,S^{0}\,X^{+1}\,X^{+2}$ (SEQ ID NO:13), wherein S^{0} is phosphorylated;

where X^{-4} and X^{+2} are independently selected from the group consisting of: an amino acid residue and an amino acid residue comprising the fluorescent label; and

where X^{-1} and X^{+1} are independently selected from the group consisting of: a hydrophobic amino acid residue and an amino acid residue comprising the fluorescent label.

63 The composition of claim **60**, wherein the labeled polypeptide is any one of **P1-P12** (SEQ ID NOs:1-12) in which the serine residue is phosphorylated.

64 The composition of claim **63**, wherein the second polypeptide comprises a 14-3-3 domain.

65 The composition of claim **64**, wherein the labeled polypeptide is **P5** and the quencher is Rose Bengal, the labeled polypeptide is **P9** and the quencher is Aniline Blue WS, the labeled polypeptide is **P2** and the quencher is Ponceau S, or the labeled polypeptide is **P12** and the quencher is Acid Green 27.

66 The composition of claim **60**, wherein the quencher forms a non-covalent complex with the labeled polypeptide, which complex is disrupted upon binding of the second polypeptide to the labeled polypeptide, thereby resulting in the increased intensity of fluorescent emission from the label.

67 The composition of claim **60**, wherein the quencher forms a non-covalent complex with the labeled polypeptide with an apparent K_d of about 20 μM or less, about 10 μM or less, or about 1 μM or less.

68 The composition of claim **60**, wherein the increased intensity of fluorescent emission from the label is an increase of at least about 7 fold, at least about 20 fold, at least about 50 fold, at least about 100 fold, or at least about 200 fold.

69 The composition of claim **60**, wherein the label is pyrene or a coumarin derivative.

70 The composition of claim **60**, wherein the quencher is selected from the group consisting of: Evans Blue, Reactive Blue, Eriochrome Black T, Alizarin Red, Aniline Blue WS, Chlorazol Black, Ponceau S, Rose Bengal, Tartrazine, Trypan Blue, and Acid Green 27.

71 The composition of claim 60, wherein when the labeled polypeptide is not bound to the second polypeptide, the quencher quenches fluorescent emission by the label by at least about 40%, as compared to fluorescent emission in the absence of the quencher.

72 The composition of claim 60, wherein the molar ratio of the quencher to the labeled polypeptide in the composition is at least about 5 to 1, at least about 10 to 1, at least about 25 to 1, or at least about 50 to 1.

73 The composition of claim 60, comprising an inhibitor or potential inhibitor of the interaction between the first and second polypeptides.

74 The composition of claim 60, wherein the labeled polypeptide comprises one or more photolabile caging groups covalently bound to the first polypeptide, which caging groups inhibit or prevent the first polypeptide from binding to the second polypeptide.

75 A method of assaying an intermolecular interaction between a first polypeptide and a second polypeptide, the method comprising:

providing a labeled polypeptide comprising the first polypeptide and a fluorescent label;

providing a quencher, wherein the quencher is not covalently bound to the first polypeptide or to the second polypeptide;

contacting the labeled polypeptide, the quencher, and the second polypeptide, thereby permitting the first polypeptide to bind to the second polypeptide, wherein binding of the first polypeptide to the second polypeptide results in an increased intensity of fluorescent emission from the label;

detecting the increased intensity of fluorescent emission from the label; and

correlating the increased intensity of fluorescent emission from the label to binding of the first and second polypeptides.

76 The method of claim 75, wherein the first polypeptide is a proline rich polypeptide and the second polypeptide comprises an SH3 domain, wherein the first polypeptide comprises a phosphorylated serine residue and the second polypeptide comprises a 14-3-3 domain, wherein the first polypeptide comprises a phosphorylated tyrosine residue and the second polypeptide comprises an SH2 or PTB domain, wherein the first polypeptide comprises a methylated lysine residue and the second polypeptide comprises a chromodomain, or wherein

the first polypeptide comprises an acetylated lysine residue and the second polypeptide comprises a bromodomain.

77 The method of claim 75, wherein the labeled polypeptide comprises amino acid sequence $X^{-4}\,R^{-3}\,R^{-2}\,X^{-1}\,S^{0}\,X^{+1}\,X^{+2}$ (SEQ ID NO:13), wherein S^{0} is phosphorylated;

where X^{-4} and X^{+2} are independently selected from the group consisting of: an amino acid residue and an amino acid residue comprising the fluorescent label; and

where X^{-1} and X^{+1} are independently selected from the group consisting of: a hydrophobic amino acid residue and an amino acid residue comprising the fluorescent label.

78 The method of claim 75, wherein the labeled polypeptide is any one of **P1-P12** (SEQ ID NOs:1-12) in which the serine residue is phosphorylated.

79 The method of claim 78, wherein the second polypeptide comprises a 14-3-3 domain.

80 The method of claim 79, wherein the labeled polypeptide is **P5** and the quencher is Rose Bengal, the labeled polypeptide is **P9** and the quencher is Aniline Blue WS, the labeled polypeptide is **P2** and the quencher is Ponceau S, or the labeled polypeptide is **P12** and the quencher is Acid Green 27.

81 The method of claim 75, wherein the quencher forms a non-covalent complex with the labeled polypeptide, which complex is disrupted upon binding of the second polypeptide to the labeled polypeptide, thereby resulting in the increased intensity of fluorescent emission from the label.

82 The method of claim 75, wherein the quencher forms a non-covalent complex with the labeled polypeptide with an apparent K_d of about 20 μM or less, about 10 μM or less, or about 1 μM or less.

83 The method of claim 75, wherein the increased intensity of fluorescent emission from the label is an increase of at least about 7 fold, at least about 20 fold, at least about 50 fold, at least about 100 fold, or at least about 200 fold.

84 The method of claim 75, wherein the label is pyrene or a coumarin derivative.

85 The method of claim 75, wherein the quencher is selected from the group consisting of: Evans Blue, Reactive Blue, Eriochrome Black T, Alizarin Red, Aniline Blue WS, Chlorazol Black, Ponceau S, Rose Bengal, Tartrazine, Trypan Blue, and Acid Green 27.

86 The method of claim 75, wherein when the labeled polypeptide is not bound to the second polypeptide, the quencher quenches fluorescent emission by the label by at least about 40%, as compared to fluorescent emission in the absence of the quencher.

87 The method of claim 75, comprising contacting the second polypeptide with a test compound, assaying the interaction between the first and second polypeptides in the presence of the test compound, and comparing the interaction between the first and second polypeptides in the presence of the test compound with interaction between the first and second polypeptides in the absence of the test compound.

88 The method of claim 75, wherein the labeled polypeptide comprises one or more caging groups associated with the first polypeptide, which caging groups inhibit the first polypeptide from binding to the second polypeptide, the method comprising uncaging the first polypeptide.

89 A composition comprising a labeled polypeptide, the labeled polypeptide comprising a fluorescent label and a polypeptide that comprises amino acid sequence $X^{-4}\,R^{-3}\,R^{-2}\,X^{-1}\,S^{0}\,X^{+1}\,X^{+2}$ (SEQ ID NO:13);

where X^{-4} and X^{+2} are independently selected from the group consisting of: an amino acid residue and an amino acid residue comprising the fluorescent label; and

where X^{-1} and X^{+1} are independently selected from the group consisting of: a hydrophobic amino acid residue and an amino acid residue comprising the fluorescent label.

90 The composition of claim 89, wherein the labeled polypeptide is any one of **P1-P12** (SEQ ID NOs:1-12).

91 The composition of claim 89, wherein the label is pyrene or a coumarin derivative.

92 The composition of claim 89, comprising a quencher.

93 The composition of claim 92, wherein the quencher is selected from the group consisting of: Evans Blue, Reactive Blue, Eriochrome Black T, Alizarin Red, Aniline Blue WS, Chlorazol Black, Ponceau S, Rose Bengal, Tartrazine, Trypan Blue, and Acid Green 27.

94 The composition of claim 89, comprising **P5** and Rose Bengal, **P9** and Aniline Blue WS, **P2** and Ponceau S, or **P12** and Acid Green 27.

95 The composition of claim 89, comprising a 14-3-3 domain.

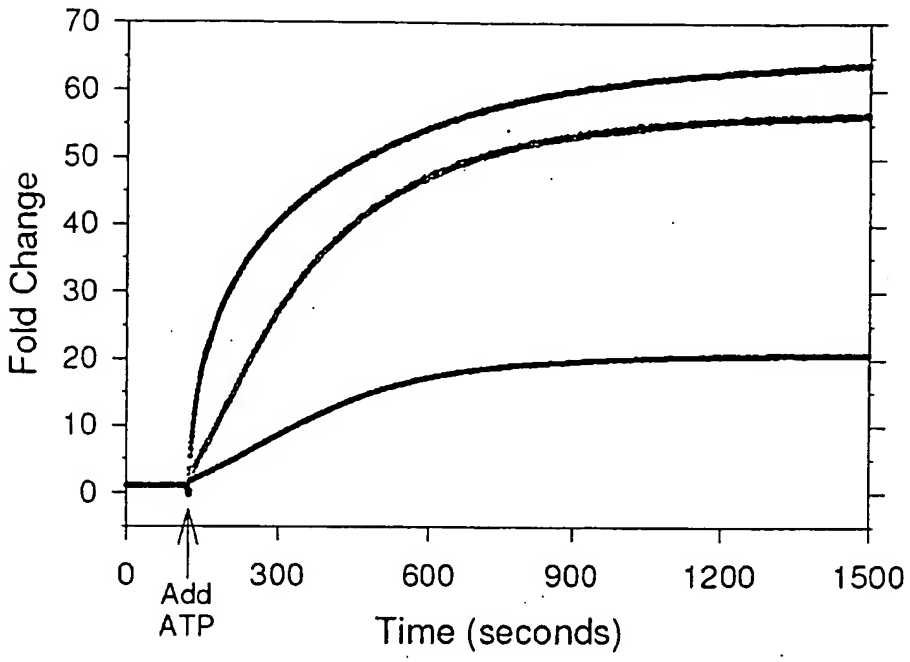

70
60
50
40
30
20
10
0
Fold Change
0
300
600
900
1200
1500
Add
ATP
Time (seconds)

FIGURE 1

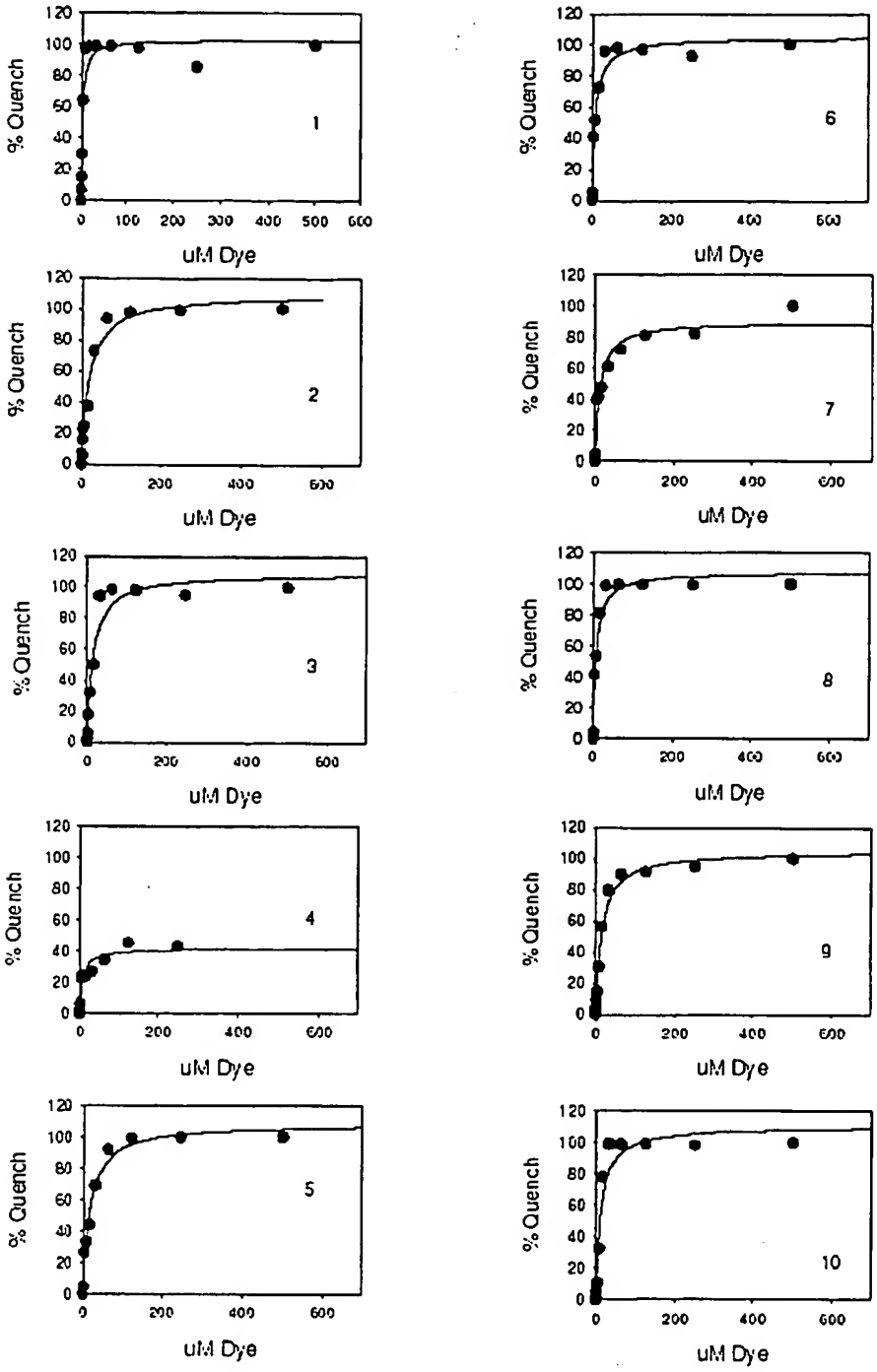
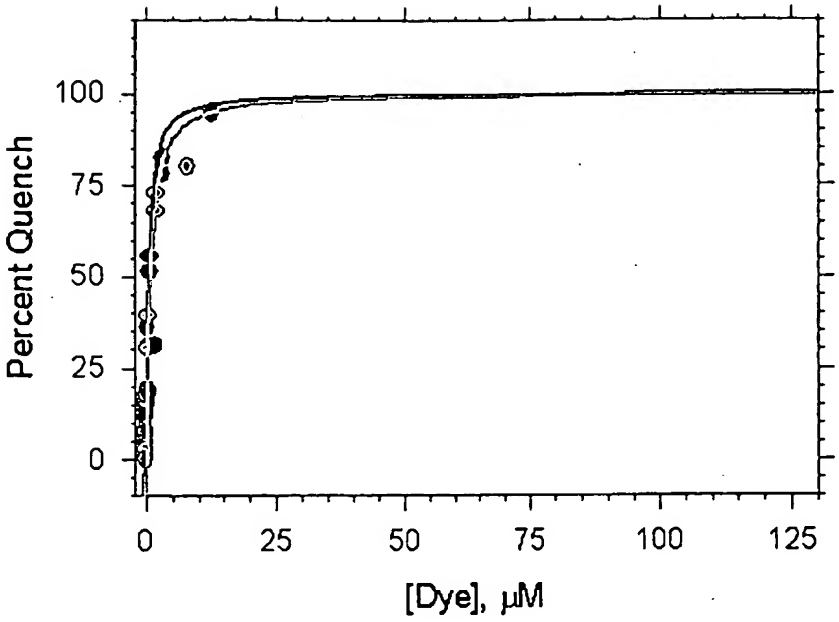


FIGURE 2

A)



B)

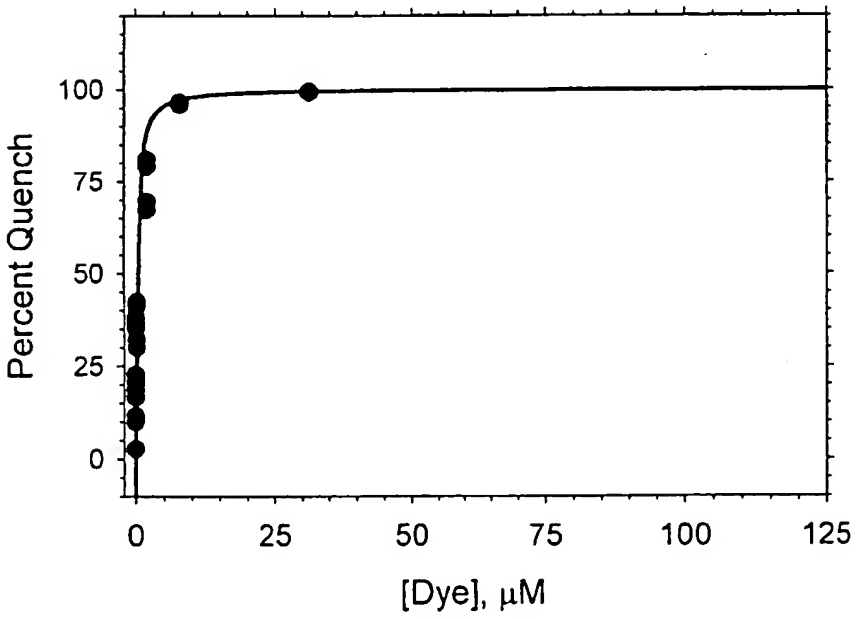


FIGURE 3A-3B

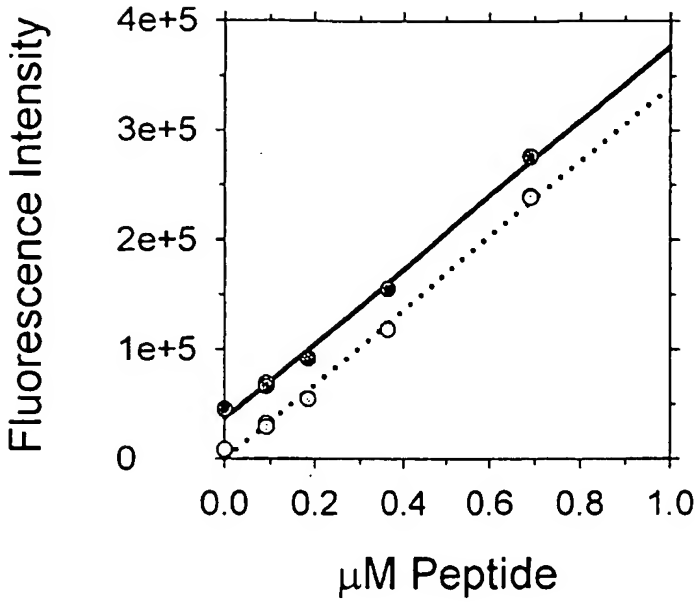

Fluorescence Intensity
4e+5
3e+5
2e+5
1e+5
0
0.0
0.2
0.4
0.6
0.8
1.0
μM Peptide

FIGURE 4

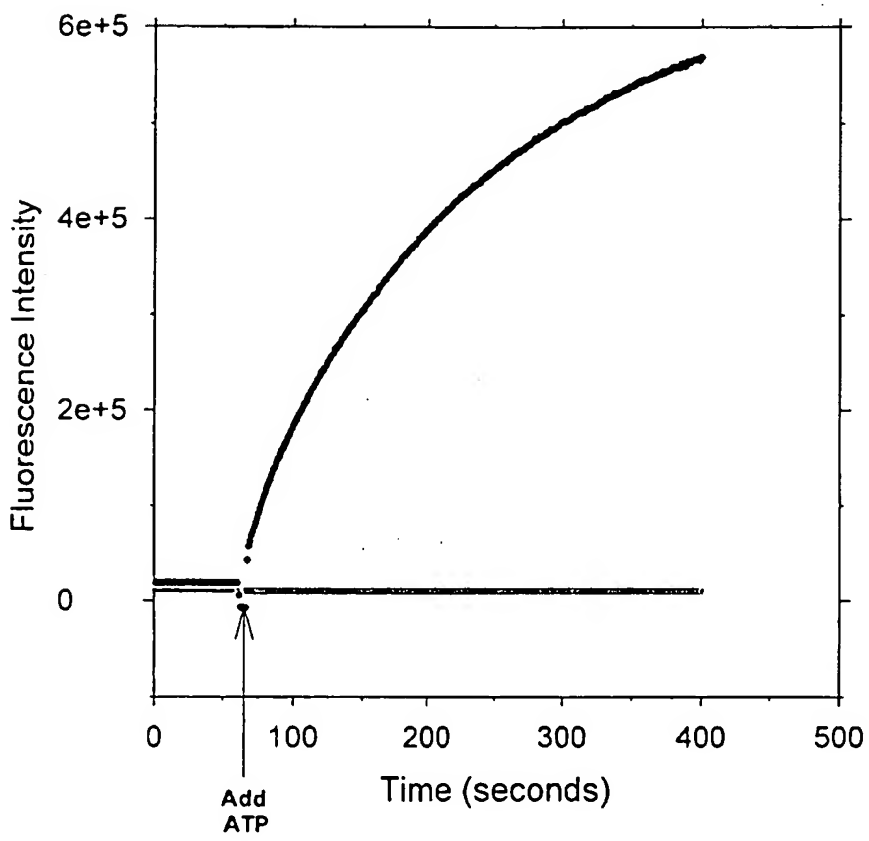
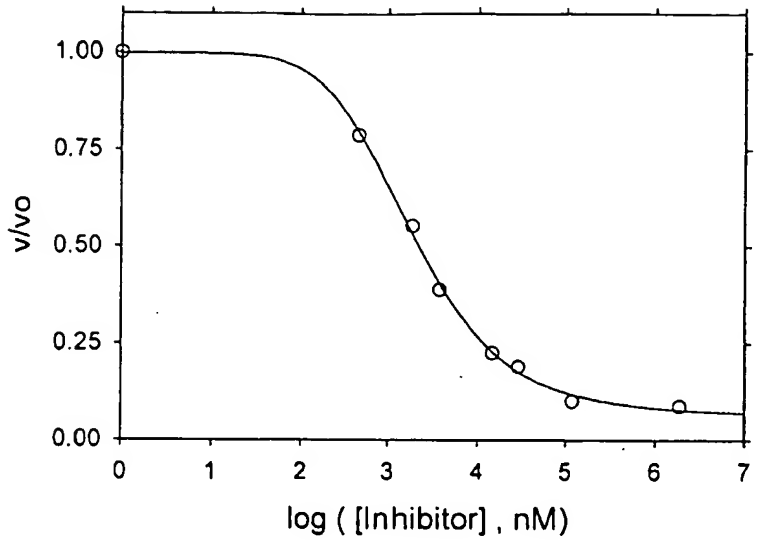

6e+5
4e+5
2e+5
0
Fluorescence Intensity
0
100
200
300
400
500
Time (seconds)
Add
ATP

FIGURE 5

A)



B)

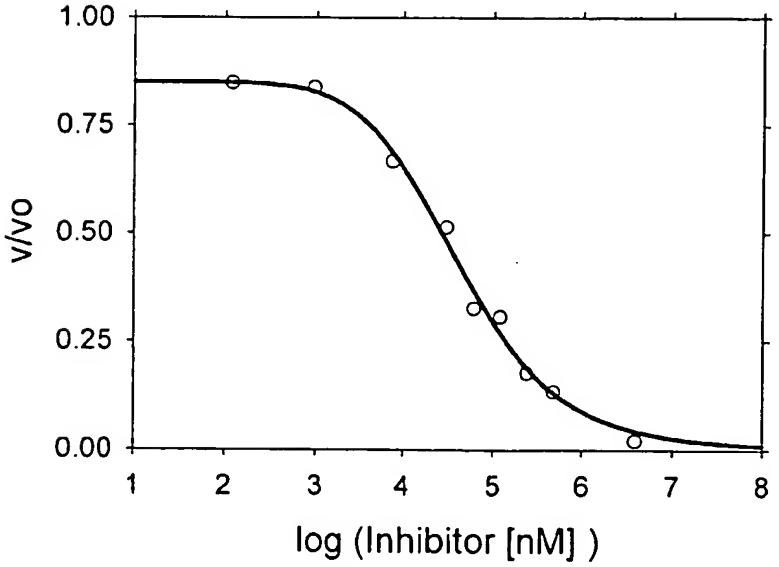
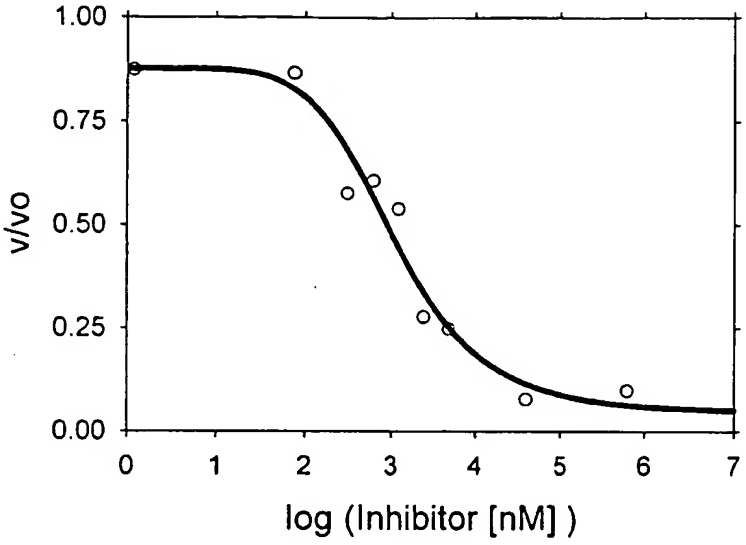


FIGURE 6A-6B

C)



D)

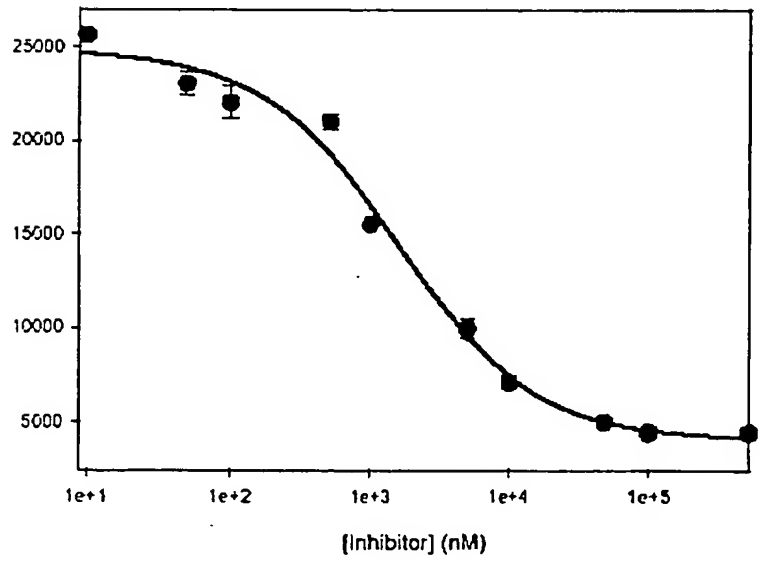


FIGURE 6C-6D

Peptide / Dye	Fold excess dye	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
D3 Evans Blue	50	1.15	0.94	0.99	0.91	0.78	0.70	0.81	0.88	0.83	0.73	0.74
	25	1.46	1.01	1.06	0.89	0.84	0.06	0.45	0.89	0.26	0.59	0.25
	10	1.43	1.37	1.06	0.91	0.92	0.74	0.31	0.36	0.24	0.64	0.12
	5	1.69	1.92	1.10	0.98	1.33	1.50	1.01	1.34	1.09	0.69	0.46
D6 Reactive Blue	50	3.85	6.30	1.68	4.27	5.79	4.70	5.20	5.01	4.03	3.48	2.46
	25	2.92	5.66	3.56	3.55	6.44	4.78	4.43	5.33	4.95	3.95	3.09
	10	1.57	2.08	2.29	2.66	3.99	2.33	3.68	3.40	3.61	3.80	1.72
	5	1.00	1.99	1.48	2.16	2.28	1.63	2.64	2.57	3.39	5.12	1.57
D18 Eriochrome Black T	50	1.10	3.71	0.31	0.59	1.12	1.52	0.48	0.45	0.65	0.72	0.30
	25	0.41	1.13	0.58	0.59	0.84	0.87	1.58	0.62	1.46	0.79	0.56
	10	1.13	1.42	1.02	1.12	1.73	1.37	1.64	1.47	1.52	2.23	0.71
	5	0.96	1.46	1.10	1.32	1.61	1.37	1.82	1.65	2.09	2.51	1.42
D19 Alizarin Red	50	1.13	1.30	1.05	1.07	1.50	1.27	1.34	1.64	2.03	2.44	1.14
	25	1.01	1.37	1.17	1.23	1.49	1.32	1.60	1.59	1.91	1.95	1.28
	10	0.96	1.38	1.21	1.33	1.59	1.27	1.63	1.57	1.98	1.90	1.35
	5	0.98	1.59	1.28	1.36	1.70	1.35	1.71	1.68	2.01	1.97	1.31
D27 Aniline Blue WS	50	1.55	1.83	0.99	1.40	1.23	1.28	1.39	1.35	1.28	1.09	1.63
	25	4.13	2.52	3.79	5.58	6.04	5.60	4.76	1.81	3.59	2.39	2.21
	10	0.47	2.48	2.17	4.82	5.36	3.48	6.63	6.22	8.94	6.77	1.78
	5	1.16	1.85	1.50	2.34	3.02	2.35	3.85	4.07	6.22	6.82	1.62
D34 Chlorazol Black E	50	0.10	0.35	0.25	0.87	0.25	0.38	0.95	0.97	0.85	0.89	0.19
	25	0.49	0.60	0.92	0.55	0.21	1.10	1.08	1.05	0.88	0.74	0.86
	10	2.44	2.84	0.71	0.93	1.52	2.32	2.49	2.83	1.61	1.32	0.83
	5	1.42	2.26	1.79	4.01	5.62	2.27	4.81	6.65	3.63	4.80	1.62
D42 Ponceau S	50	3.37	6.57	2.88	2.69	4.36	1.77	1.27	3.26	4.02	2.81	2.38
	25	1.84	4.61	2.75	2.88	3.23	3.13	2.19	2.01	4.64	5.66	2.12
	10	1.76	1.92	2.26	2.63	3.30	3.36	1.92	3.86	4.83	7.99	2.06
	5	1.35	1.93	1.81	2.26	2.85	2.77	2.00	3.14	4.32	7.56	2.10
D43 Rose Bengal (Cert)	50	0.96	0.72	0.97	0.91	0.95	0.94	0.85	0.95	0.98	0.89	0.84
	25	1.03	1.58	1.13	1.10	1.02	1.20	0.85	0.81	1.11	1.23	0.32
	10	2.63	2.87	1.98	2.88	3.45	3.28	1.12	2.19	1.85	1.32	0.94
	5	1.20	2.58	2.70	6.52	8.36	2.71	7.44	5.43	3.51	3.26	3.54
D47 Tartrazine	50	1.31	2.51	1.02	1.47	2.05	1.25	1.65	1.79	1.53	1.92	1.43
	25	1.16	1.82	1.47	1.46	1.79	1.42	1.77	1.58	1.82	1.76	1.28
	10	1.06	1.61	1.34	1.42	1.52	1.27	1.55	1.47	1.57	1.64	1.17
	5	0.99	1.41	1.19	1.28	1.43	1.23	1.46	1.39	1.55	1.47	1.15
D48 Trypan Blue	50	2.60	6.03	1.60	2.76	3.48	3.34	5.62	3.52	2.62	3.39	3.17
	25	3.40	5.11	1.91	1.72	3.61	3.05	4.10	4.03	3.67	3.53	2.45
	10	3.04	2.81	2.72	2.18	2.72	2.17	4.76	4.87	3.96	3.45	3.40
	5	1.30	2.20	2.23	3.03	6.21	3.54	7.15	6.14	7.18	7.22	3.06

FIGURE 7

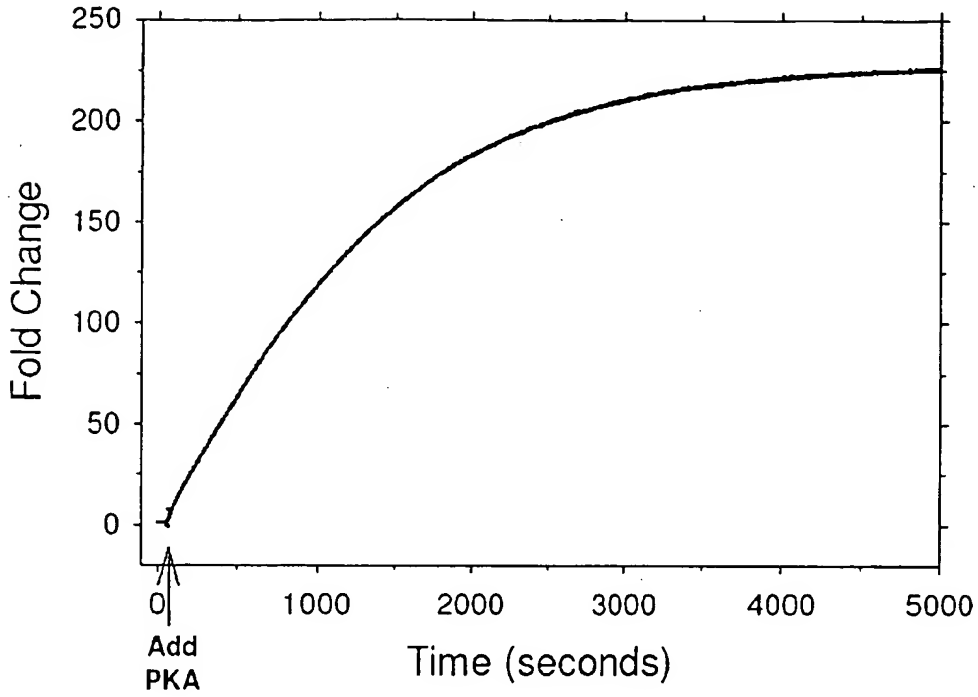

250
200
150
100
50
0
Fold Change
0
1000
2000
3000
4000
5000
Add
PKA
Time (seconds)

FIGURE 8